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NUTRITIONAL REGULATION OF LEPTIN IN DAIRY CATTLE

By

PRASANTH K. CHELIKANI ©

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

ANIMAL SCIENCE

DEPARTMENT OF AGRICULTURAL, FOOD AND NUTRITIONAL SCIENCE
UNIVERSITY OF ALBERTA
EDMONTON, ALBERTA

FALL 2003

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Dated: July 25th, 2003

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled NUTRITIONAL REGULATION OF LEPTIN IN DAIRY CATTLE submitted by PRASANTH K. CHELIKANI in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in ANIMAL SCIENCE.

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ABSTRACT

Leptin, a hormone secreted primarily from white adipose tissue, plays a role in the regulation of energy balance and reproduction. Studies were conducted to determine the tissue distribution of leptin and leptin receptor and to examine the role of nutrition in the regulation of leptin secretion in Holstein cattle.

In male calves, leptin mRNA was detectable in adipose tissue with similar transcript abundance among adipose depots. The mRNA for the long-form of the leptin receptor, Ob-Rb, was detectable in a wide array of central and peripheral tissues whereas the short-form, Ob-Ra, was expressed in only a few tissues.

Plasma leptin concentrations were acutely responsive to short-term fasting and refeeding but the response differed among cattle of varying physiological states. The fasting-induced hypoleptinemia was delayed in non-lactating cows and postpubertal heifers compared to the rapid response observed in early lactation cows.

Rumen and duodenally cannulated cows were used to determine the effects of canola oil supplementation on leptin and cholecystokinin (CCK) gene expression and release. Both feeding and abomasal infusion of canola oil resulted in a similar increase in gene expression and plasma concentrations of CCK but only infusion caused a reduction in feed intake, which is suggestive of a paracrine/neurocrine role for CCK in mediating satiety. Neither body condition score (BCS) nor plasma leptin concentrations were altered with fat supplementation, indicating that leptin does not mediate fat-induced satiety and/or that the leptin response to fat is dependent on BCS.

The leptin response to lipid or glucose supplementation was determined in early and late- lactation cows. Late lactation cows, with high BCS, exhibited a robust leptin

response to parenteral glucose or lipid infusions, whereas early lactation cows, with low BCS, were unresponsive. In comparison to the direct rapid effects of lipid infusion on leptin secretion, the glucose-induced hyperleptinemia was delayed and was preceded by a strong insulin response in late-lactation cows.

Long-term changes in body composition and plasma leptin concentrations were determined in heifer calves fed diets varying in energy and protein density. Puberty occurred at a constant body composition and body weight independent of growth rate. Plasma leptin concentrations increased with approaching puberty suggesting that leptin might relay information on body reserves to the brain to initiate puberty in heifers.

In summary, leptin is secreted from adipose tissue and may have multiple physiological functions in cattle. In the short-term, the response of leptin to feed deprivation or nutrient infusions is dependent on physiological state and body fat reserves. In the long-term, leptin secretion reflects changes in body weight and adipose tissue reserves.

ACKNOWLEDGEMENTS

The research work presented here would not have been successfully completed but for the cooperation and support of the members of my supervisory committee, the dairy research group, colleagues, and family members.

The guidance, support, and encouragement from Dr. John J. Kennelly, my supervisor, were invaluable. I am indebted to him for giving me the freedom to do this work and for setting me on my research career.

I am grateful to the members of my supervisory committee- Dr. Divakar J. Ambrose and Dr. Timothy J. Kieffer for their interest, advice, and encouragement, during the course of my doctoral program. I would also like to thank Dr. Yves Chilliard and Dr. Victoria J. Harber for serving on my final examining committee.

My thanks to Dr. Duane H. Keisler for collaborating and helping with the leptin and growth hormone assays.

I appreciate the support of the dairy research group for their help during various stages of my research. I am specially thankful to Dr. John Bell, Jeff Kaufman, Dr. David Glimm, Dr. Reza Khorasani, Dr. Lorraine Doeppel, Naomi Beswick, Pavol Zalkovic, and staff of the dairy research unit for their input, suggestions, and their help during the experiments and in analyses.

I would also like to thank Pat Marceau for her help with analyses of the large number of feed, digesta, and blood samples. I am thankful to Shirley Shostak for introducing me to radioimmunoassay techniques and for helping me with the hormone assays.

I am grateful to my parents Tara Devi and Dharma Rao, wife Indira, and brother Prashen, for their constant loving support and encouragement. The company of my six month old son, Vijay, during the drafting of this thesis deserve special mention.

Financial support was provided by Alberta Agricultural Research Institute, Alberta Milk, and the FS Chia PhD Scholarship by the University of Alberta.

TABLE OF CONTENTS

		Page
CHAPTER 1	**Literature review**	**1**
	1.1 Introduction	1
	1.2 Leptin, leptin receptors and leptin transport	2
	1.3 Leptin in circulation	6
	1.4 Regulation of leptin production	10
	1.5 Role of leptin in the regulation of feed intake and puberty	27
	1.6 Conclusion	34
	1.7 Research Objectives	34
	1.8 Literature cited	44
CHAPTER 2	**Tissue distribution of leptin and leptin receptor mRNA in the bovine**	**70**
	2.1 Introduction	70
	2.2 Materials and methods	70
	2.3 Results and Discussion	72
	2.4 Literature cited	78
CHAPTER 3	**Effect of short-term fasting on plasma concentrations of leptin and other hormones and metabolites in dairy cattle**	**80**
	3.1 Introduction	80
	3.2 Materials and methods	81
	3.3 Results	86
	3.4 Discussion	89
	3.5 Literature cited	101
CHAPTER 4	**Effect of feeding or abomasal infusion of canola oil on nutrient digestion, milk composition, mRNA abundance and plasma concentrations of leptin and cholecystokinin in dairy cows**	**105**
	4.1 Introduction	105

	4.2 Materials and methods	106
	4.3 Results	114
	4.4 Discussion	118
	4.5 Literature cited	145
CHAPTER 5	**Effect of isoenergetic jugular infusion of lipid or glucose on plasma concentrations of leptin and other hormones and metabolites in early and late- lactation dairy cows**	**151**
	5.1 Introduction	151
	5.2 Materials and methods	152
	5.3 Results	156
	5.4 Discussion	159
	5.5 Literature cited	179
CHAPTER 6	**Effect of dietary energy and protein density on body composition, attainment of puberty, ovarian follicular dynamics and, peripubertal changes in plasma concentrations of leptin and other hormones and metabolites in dairy heifers**	**184**
	6.1 Introduction	184
	6.2 Materials and methods	185
	6.3 Results	191
	6.4 Discussion	196
	6.5. Literature cited	219
CHAPTER 7	**General discussion**	**224**
	7.1 Introduction	224
	7.2 Detection of mRNA for leptin and leptin receptor in tissues of cattle	224
	7.3 The response of plasma leptin concentrations to short-term feed deprivation is dependent on the physiological stage and body condition of dairy cattle	225
	7.4 The mRNA abundance and plasma leptin concentrations	226

are unaffected whereas both transcript abundance and plasma cholecystokinin concentrations are increased by canol oil supplementation to dairy cattle

7.5 Response of plasma leptin concentrations to jugular infusion of glucose or lipid is depedent on the stage of lactation of Holstein cows 227

7.6 Dietary energy and protein density affects plasma leptin concentrations in dairy heifers during the peripubertal period 228

7.7 Future research 229

7.7 Conclusions 231

Appendix methods

Appendix 1: Isolation of RNA from adipose tissue biopsies using TRIzol reagent 233

Appendix 2: Leptin RIA procedure 235

Appendix 3: Bovine Insulin RIA procedure 238

Appendix 4: Isolation of RNA from intestinal biopsies 242

Appendix 5: Rapid method for determination of total fatty acid content and composition of feed stuffs, digesta, feces, and plasma 244

LIST OF TABLES

		PAGE
Table 1.1	Characteristics of ruminant specific assays used for quantifying leptin concentrations in circulation	41
Table 1.2	Summary of effects of leptin on intake, hormones and metabolite secretion *in vivo*, and hormone secretion *in vitro* in farm animals	42
Table 2.1	Primer pairs for RT-PCR amplification of each target gene, annealing temperature, number of cycles of amplification, and length of PCR products	75
Table 3.1	Effect of short-term fasting and refeeding on milk yield and composition, and energy balance in dairy cows.	94
Table 3.2	Pearson correlation coefficients among plasma concentrations of leptin, glucose, NEFA, insulin, IGF-1, and GH from all three fasting-refeeding experiments	95
Table 4.1	Ingredient and chemical composition of basal diet	129
Table 4.2	Fatty acid composition of canola oil	130
Table 4.3	Effect of feeding or abomasal infusion of canola oil on ruminal and total tract digestibilities of DM, OM, CP, NDF, ADF, and cellulose.	131
Table 4.4	Effect of feeding or abomasal infusion of canola oil on ruminal fermentation characteristics.	133
Table 4.5	Effect of feeding or abomasal infusion of canola oil on intake and duodenal flow of fatty acids.	134
Table 4.6	Effect of feeding or abomasal infusion of canola oil on total tract digestibility and postruminal absorption of fatty acids	136
Table 4.7	Effect of feeding or abomasal infusion of canola oil on milk composition and yield.	137
Table 4.8	Effect of feeding or abomasal infusion of canola oil on milk fatty acid composition.	138

Table 4.9	Primer conditions for semi-quantitative RT-PCR amplification of each target gene	140
Table 4.10	Effect of feeding or abomasal infusion of canola oil on plasma glucose and plasma fatty acid composition.	141
Table 4.11	Effect of feeding or abomasal infusion of canola oil on fatty acid composition of subcutaneous adipose tissue.	142
Table 5.1	Ingredient and chemical composition of basal TMR on DM basis	165
Table 5.2	Effect of intrajugular infusions of either saline, dextrose or Intralipid on DMI, BW, BCS and, milk yield and composition in early and late lactation Holstein cows.	166
Table 5.3	Effect of intrajugular infusions of either saline, dextrose, Intralipid on plasma concentrations of glucose, NEFA, BHBA, leptin, insulin, IGF-1, and GH in early and late lactation Holstein cows	167
Table 5.4	Area under the curve for profiles of hormones and metabolites of Holstein cows receiving jugular infusions of either saline, dextrose, or Intralipid in early and late lactation.	169
Table 6.1	Ingredient and chemical composition of diets	202
Table 6.2	Effect of dietary energy and protein density on average daily gain, age, body weight, body condition score, back-fat thickness, empty body fat and protein percent, and growth measurements at puberty.	204
Table 6.3	Ovarian follicular characteristics in prepubertal heifers at 8 mo of age based on ultrasonographic ovarian scans over a 21 d period.	205
Table 6.4	Ovarian follicular characteristics in heifers during the peripubertal period.	206
Table 6.5	Characteristics of the CL and progesterone concentrations heifers fed high, or medium energy- and protein dense diets	208

LIST OF FIGURES

		PAGE
Figure 1.1	Role of leptin in the regulation of food intake and puberty in monogastric species.	36
Figure 1.2	Genomic structure of the bovine leptin gene	37
Figure 1.3	Comparison of nucleotide sequences of leptin from domestic animals, humans, rodents and birds	38
Figure 1.4	Phylogenetic tree for leptin	39
Figure 2.1	Detection of mRNA corresponding to leptin, the long-form and short- forms of the leptin receptor, GAPDH, in various peripheral and central tissues of a typical male Holstein calf by RT-PCR	76
Figure 2.2	Semiquantitative RT-PCR for determining relative abundance of leptin mRNA in adipose depots of male Holstein calves	77
Figure 3.1	Effect of short term fasting and refeeding on feed intake and body weight in lactating dairy cows, non-lactating pregnant dairy cows, and postpubertal dairy heifers.	97
Figure 3.2	Plasma concentrations of leptin, NEFA, insulin, glucose, IGF-1, and GH in early lactation cows fasted for 48 h and subsequently refed	98
Figure 3.3	Plasma concentrations of leptin, NEFA, insulin, glucose, IGF-1, and GH in non-lactating pregnant cows fasted for 48 h and subsequently refed	99
Figure 3.4	Plasma concentrations of leptin, NEFA, insulin, glucose, IGF-1, and GH in postpubertal heifers fasted for 72 h and subsequently refed	100
Figure 4.1	Effect of feeding or abomasal infusion of canola oil on temporal changes in plasma concentrations of CCK, leptin, insulin, and IGF-1.	143
Figure 4.2	Effect of feeding or abomasal infusion of canola oil on	144

	mRNA abundance of CCK and adipose tissue genes analyzed by semi-quantitative RT-PCR	
Figure 5.1	Plasma concentrations of glucose in Holstein cows infused intrajugularly with either saline, dextrose, or Intralipid in early and late lactation	170
Figure 5.2	Plasma concentrations of NEFA in Holstein cows infused intrajugularly with either saline, dextrose, or Intralipid in early and late lactation	171
Figure 5.3	Plasma concentrations of BHBA in Holstein cows infused intrajugularly with either saline, dextrose, or Intralipid in early and late lactation	172
Figure 5.4	Plasma concentrations of insulin in Holstein cows infused intrajugularly with either saline, dextrose, or Intralipid in early and late lactation	173
Figure 5.5	Plasma concentrations of leptin in Holstein cows infused intrajugularly with either saline, dextrose, or Intralipid in early and late lactation	174
Figure 5.6	Plasma concentrations of IGF-1 in Holstein cows infused intrajugularly with either saline, dextrose, or Intralipid in early and late lactation	175
Figure 5.7	Plasma concentrations of GH in Holstein cows infused intrajugularly with either saline, dextrose, or Intralipid in early and late lactation	176
Figure 5.8	Proposed mechanisms for the effects of glucose and lipid on leptin secretion in cattle	177
Figure 6.1	Schematic overview of experimental design	210
Figure 6.2	Changes in estimated body fat and body protein percent from 8 to 12 mo in heifers fed high, medium, or low, energy and protein dense diets.	211
Figure 6.3	Diameter profiles of nonovulatory dominant follicle at 8	212

	mo of age in heifers fed high, medium, or low energy and protein dense diets. Diameter profiles of the nonovulatory dominant follicle prior to first ovulation, and the first ovulatory follicle in heifers fed diets varying in energy and protein density	
Figure 6.4	Effect of feeding high, medium, or low energy and protein dense diets on plasma LH characteristics in dairy heifers	213
Figure 6.5	Plasma leptin concentrations in heifers fed diets varying in energy and protein density	214
Figure 6.6	Effect of feeding high, medium, or low energy and protein dense diets on plasma leptin characteristics in dairy heifers	215
Figure 6.7	Changes in body weight and back-fat thickness in heifers fed diets varying in energy and protein density.	216
Figure 6.8	Changes in plasma concentrations of glucose, insulin, and NEFA, in heifers fed diets varying in energy and protein density	217
Figure 6.9	Changes in plasma concentrations of glucose, insulin, NEFA, in heifers fed diets varying in energy and protein density	218

LIST OF ABBREVIATIONS

ACC	Acetyl coA carboxylase
ADF	Acid detergent fiber
AUC	Area under the curve
bp	Base pair
BCS	Body condition score
BHBA	Betahydroxy butyrate
CCK	Cholecystokinin
cDNA	Complementary cDNA
CP	Crude protein
CL	Corpus luteum
CLA	Conjugated linoleic acid
DIM	Days in milk
DMI	Dry matter intake
FAME	Fatty acid methyl esters
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GH	Growth hormone
IGF-1	Insulin-like growth factor-1
LH	Luteinizing hormone
mRNA	Messenger RNA
NDF	Neutral detergent fiber
NEFA	Nonesterified fatty acids
OD	Optical density units
OM	Organic matter
RIA	Radioimmunoassay
RT-PCR	Reverse transcription-polymerase chain reaction
SEM	Standard error of mean
TMR	Total mixed ration
VFA	Volatile fatty acids

CHAPTER 1

Literature Review

1.1. Introduction

Feed intake is a key factor influencing the productivity and efficiency of domestic animals. An understanding of the factors controlling feed intake is critical to improving the efficiency of milk and meat production in cattle. In the case of dairy cattle, feed intake is particularly critical in early lactation. The amount of feed consumed during early lactation will determine the extent of body weight loss, which in turn influences milk production and reproductive performance. During the prepubertal period, the plane of nutrition determines the extent of build-up of body reserves and mammary development, thereby influencing the timing of puberty and milk production potential.

Though some knowledge has been gained about the physical and chemical factors influencing feed intake, the role of hormones has only recently come under investigation. Among the gut hormones, the role of cholecystokinin (CCK) in regulating feed intake in cattle has been documented (Choi et al., 2000; Suominen et al., 1998). An assumption in current models of feed intake regulation in cattle is that adipose tissue is a passive energy depot, without any direct effect on feed intake. However, recent studies in rodents and humans challenge this concept. There is now evidence that fat stores play a critical role in regulating feed intake by secreting a potent satiety hormone – leptin. The current understanding of leptin physiology is that leptin is secreted primarily from white adipose tissue into blood and transported to the brain where it modulates the activity of other neuropeptides (primarily NPY) in such a way as to reduce food intake and increase energy expenditure (Houseknecht et al., 1998). It is also believed that leptin is an important signal linking nutritional status to reproductive function (Chehab et al., 1997). Because leptin is synthesized and secreted primarily by adipose tissue, the abundance of leptin mRNA and protein in this tissue, as well as plasma leptin levels, correlate strongly with, and are an index of, the total amount of fat tissue in humans and rodents (Maffei et al., 1995). Therefore, it is hypothesized that during states of negative energy balance the

decrease in adipose tissue mass and leptin secretion favors increased food intake and energy storage. Conversely, under positive energy states the rising leptin levels inhibit food intake (Woods et al., 1998). An overview of the role of leptin in the regulation of food intake and puberty in monogastrics is presented in Figure 1.1. To date, much of the research on leptin physiology has been conducted in rodents and humans. The importance of leptin in the regulation of feed intake, reproduction and other neuroendocrine adaptations is just being addressed in domestic animals. Because of the relative scarcity of information on leptin in ruminants, this review will rely to a major extent on data from rodent and human studies. The cloning and tissue expression of leptin and leptin receptors, the nutritional regulation of leptin, and the role of leptin in the regulation of food (feed) intake and puberty are reviewed here.

1.2 Leptin, Leptin receptors, and leptin transport

1.2.1 *The OB (Lep) gene and its product, Leptin*

1.2.1.1. *Cloning of the leptin gene*

The importance of adipose tissue reserves in the regulation of food intake and puberty was first recognized by Kennedy (1953). According to the 'adipostat' model proposed by Kennedy (1953), signals relating to fat reserves inform the brain about the energy status and any change in energy balance will lead to changes in food intake and energy expenditure to maintain a stable body weight. It was also hypothesized that the signal from adipose reserves is responsible for initiation of hypothalamic control of ovarian function and puberty (Kennedy and Mitra, 1963). Later, based on studies on parabiosis of normal rats with obese rats containing a lesion in the ventromedial hypothalamus, Hervey (1958) concluded that a circulating factor from the lesioned obese rat inhibited food intake in the normal rat.

In December 1994, Friedman's group at Rockfellar University (Zhang et al., 1994) identified the putative 'satiety signal', as a 167 amino acid protein product of the *obese (ob)* gene which was later named leptin (Greek: *leptos* = thin). Since this landmark paper, homologs of the *ob* gene have been cloned in a number of domestic species including cattle (Ji et al., 1998), sheep (Dyer et al., 1997a), pigs (Ramsay et al., 1998) and

chicken (Taouis et al., 1998). The bovine leptin gene has been mapped to chromosome 4 (Pomp et al., 1997), and similar to other species, the gene (Taniguchi et al., 2002) spans 18.9 kb and consists of 3 exons seperated by 2 introns (Figure 1.2). The coding region is located in exons 2 and 3, and the promoter contains binding sites for transcription factors such as CCAAT/enhancer binding protein (C/EBP) and Sp1. Bovine leptin exhibits 87% homology to human and mouse leptins, and 92% and 95% homology to pig and sheep leptins, respectively (Ji et al., 1998). For comparison purposes, the nucleotide sequences of leptin from cattle, sheep, goat, pig, horse, human, mouse, and chicken are aligned using Clustal W and shown in Figure 1.3. These nucleotide sequences were used to generate a phylogenetic tree using the neighbour-joining method (UGPMA method version 3.573c; Bioedit program). An unrooted tree showing the evolution of leptin is shown in Figure 1.4.

Mutations in the *ob* gene have been associated with obesity in rodents and humans. In the ob^{2J}/ob^{2J} mice a mutation in the first intron results in the absence of the leptin mRNA production (Zhang et al., 1994). In humans deletion of a single guanine nucleotide in codon 133 leads to a frameshift mutation and production of truncated leptin protein that is rapidly degraded (Montague et al., 1997a). Similarly, cattle homozygous for Thymine at the first base of codon 25 were found to have greater leptin mRNA and higher carcass fat than cattle homozygous for Cytosine at the same position (Buchanan et al., 2002). However, the direct functional consequences of mutations in the bovine leptin gene remain to be determined.

1.2.1.2. *Leptin structure and tissue expression*

Analysis of the crystal structure of a mutant form of the human leptin protein (E-100) by Zhang et al. (1997) revealed a four-helix bundle structure similar to other cytokines. The protein contains four antiparallel α-helices connected by two long crossover links and one short loop arranged in a left-handed helical bundle, which forms a two layer packing. The 167 amino acid sequence of leptin contains two cysteine residues (96 and 146) that form a disulfide bond between the CD-loop and C-terminus of the protein. As mutations of these cysteine residues inactivates the protein, it was

initially believed that the disulfide bond is critical for structural folding and receptor binding of the protein. The activity of the molecule was further narrowed down to the amino acid residues 106-140 near the C-terminus (Grasso et al., 1997, 1999). In contrast to these studies, Imagawa et al. (1998a) contend that the N-terminal amino acid sequence (22-115) is essential for receptor binding and biological function whereas the C-terminal disulfide bond is not needed for the activities of leptin. Clearly, the structure-function relationships of the protein are not completely understood. The protein, however, does not undergo any post-translational modifications because the molecular mass of the native protein is identical to that predicted by the primary sequence (without the signal sequence) (Cohen et al., 1996).

Leptin is primarily synthesized by adipose tissue in both monogastrics (Ahima and Flier, 2000; Ramsay et al., 1998; Taouis et al., 1998; Ashwell et al., 1999) and ruminants (Ji et al., 1998; Dyer et al., 1997a,b). However, leptin expression has also been detected in a number of other tissues including muscle (Wang et al., 1998), brain (Morash et al., 1999), stomach (Bado et al., 1998), mammary epithelium (Aoki et al., 1999), placenta (Hoggard et al., 1998), bone (Reseland and Gordeladze, 2002) of rodents, and in the liver of chickens (Ashwell et al., 1999). In addition to adipose tissue, leptin mRNA expression in cattle was also detectable by RT-PCR in the mammary gland of adult animals (Smith and Sheffield, 2002a), and in the rumen, abomasum, and duodenum of calves before weaning (Yonekura et al., 2002). Apart from adipose tissue, leptin expression in sheep was also reported in the mammary gland (Bonnet et al., 2002), and in the brain, adipose tissue and liver of the fetus (Ehrhardt et al., 2002).

1.2.2. *The leptin receptors*

1.2.2.1. *Receptor isoforms and structure*

Tartaglia et al. (1995) first reported the cloning of the leptin receptor from the choroid plexus of a mouse cDNA library and the human homologue from a total brain library. Alternative splicing of this single receptor gene produces at least six different isoforms, Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf, with identical extracellular domains but differing in the length of the transmembrane and intracellular domains

(White and Tartaglia, 1996; Tartaglia, 1997). In all receptors, the extracellular domain, with 816 amino acids, contains two cytokine like binding motifs and a fibronectin type III domain. Except Ob-Re, other short isoforms have similar transmembrane domains, 23 amino acids long. The intracellular domain contains 303 amino acids in the long form, Ob-Rb, whereas the short forms have truncated intracellular domains ranging from 32-40 amino acids in length. The Ob-Re isoform contains only the extracellular domain with no transcellular or intracellular domains. The intracellular domains of all forms have a conserved 29 amino acid sequence containing a 'Box 1' Janus-family tyrosine kinase (JAK) binding domain whereas Ob-Rb contains an additional 'Box 2' motif and signal-transducers and activators of transcription (STAT) binding sites.

Although Ob-Rb can form homodimers independent of ligand binding, the conformation of the receptor might prevent close proximity of JAKs for signal transduction (Sweeney, 2002). Binding of leptin does not signficantly increase the number of receptors but induces a conformational change in the leptin-Ob-Rb heterodimer complex which allows JAKs to interact, become activated, and phoshporylate other JAKs and tyrosine residues on the receptor. Phosphorylation of tyrosine 1138 on Ob-Rb recruits STATs which in turn become phosphorylated and activated. Activated STATs, dissociate from the receptor complex, form homo- or heterodimers, and translocate to the nucleus to regulate gene expression. In adition to this well-defined JAK-STAT pathway (White and Tartaglia, 1996) other signalling molecules and pathways affected by Ob-Rb include JNK, p38, ERK, SHP-2, PLC, NO, DGK, PGE2/PGF2, cAMP, PDE, SOCS-3, PI3K, IRS, PKB, PKB, PKC, p70S6K, and ROS (Sweeney, 2002). Although Ob-Rb was initially believed to be the only isoform capable of signal transduction recent evidence suggests that the short-forms also have signal transduction capabilities (Bjorbaek et al., 1997; 1998; Yanagihara et al., 2000). In general, Ob-Rb is predominantly expressed in central tissues while the short-forms are more abundant in peripheral tissues of rodents and humans (Tartaglia et al., 1997).

The long-form of the leptin receptor has been cloned in domestic species including cattle (Pfister-Genskow et al., 1997), sheep (Dyer et al., 1997b), pigs (Ernst et al., 1997) and chicken (Horev et al., 2000). The gene for bovine Ob-Rb has been mapped

to chromosome 3q33 (Pfister-Genskow et al., 1997), and Ob-Rb mRNA expression has been detected in the hypothalamus, adipose tissue (Ren et al., 2002; Silva et al., 2002), pituitary, mammary parenchyma, heart, ovary, lung, liver, muscle, testis, spleen, (Silva et al., 2002) and aorta (Parhami et al., 2001) of cattle. In contrast to the almost universal distribution of the short forms of the receptors in humans and rodents, in bovines the Ob-Ra was found to be expressed only in liver, pituitary, spleen (Silva et al., 2002) and adrenal medulla (Yanagihara et al., 2000).

Among domestic animals, the expression pattern of leptin receptor in the brain has been relatively well characterised in the sheep. The Ob-Rb mRNA in sheep was detected by insitu hybridization in the arcuate, dorsomedial, and ventromedial hypothalamic nuclei (Dyer et al., 1997b; Williams et al., 1999), and in the hippocampus, cerebral cortex, preoptic area, stria terminalis, and choroid plexus (Williams et al., 1999). By double-label immunofluorescence histochemistry, with an antiserum that recognizes both Ob-Rb and Ob-Ra, immunoreactivity was found in the anterior pituitary with 69% somatotropes, 29% gonadotropes and 27% corticotropes staining positive in the para tuberalis and 90% of the gonadotropes immunopositive in the pars tuberalis (Iqbal et al., 2000). Using an antiserum specific for the intracellular domain of Ob-Rb, Iqbal et al. (2001) could detect Ob-Rb immunoreactivity in the median eminence, zona incerta, perfornical, anterior and lateral hypothalamic areas; and in the arcuate, peri and para ventricular, supraoptic, dorso and ventromedial, hypothalamic nuclei. Further, double labelling showed colocalisation of Ob-Rb in nearly 32% corticotropin-releasing factor (CRH) containing cells, 60% neuropeptide-Y (NPY) cells, 61% galanin cells, 90% pro-opiomelanocortin cells (POMC), 100% melanin concentrating hormone (MCH) and orexin cells (Iqbal et al., 2001), and 100% of somatostatin neurons in the arcuate and dorso and ventromedial hypothalamic nuclei (Iqbal et al., 2000). Thus, the widespread expression of Ob-Rb in different neuronal cell types is indicative of potential functions of the leptin system in diverse physiological processes including energy balance, stress response, reproduction and growth in ruminants.

1.3. Leptin in circulation

1.3.1. *Leptin in circulation of humans and rodents*

1.3.1.1. *Circulating leptin concentrations*

An accurate measurement of the concentrations of leptin in circulation is critical to an understanding of the physiological role of this hormone in any species. Following the discovery of leptin, radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) were rapidly developed for quantitating the hormone in circulation of humans and rodents (Considine et al., 1996; Ma et al., 1996; Landt et al., 1998; Imagawa et al., 1998b). The circulating leptin concentrations reported among lean individuals range from 1 to 16 μg/L in humans (Considine et al., 1996), 0.5 to 6 μg/L in rats (Landt et al., 1998), and 2 to 11 μg/L in mice (Ahren et al., 1997), while in obese humans the leptin concentrations were reported to be as high as 80 μg/L (Ma et al., 1996). The type of anticoagulant used for blood sampling and the storage conditions used do not seem to have a major affect on the stability of leptin in plasma or serum. For example, leptin concentrations in human serum or plasma collected using heparin, EDTA, fluoride as anticoagulants are reported to be stable for atleast 120 h at 4^0C and atleast 60 h at 30^0C (Evans et al., 2001a). While the early studies, mentioned above, detected total circulating leptin concentrations, they were unable to characterize leptin binding properties in circulation.

1.3.1.2. *Leptin binding proteins and half-life*

Binding proteins in circulation modulate the biological activity of most hormones. Several studies have shown that leptin circulates both as a free form and protein-bound form in rodents and humans, with a majority of the bound-form being associated with the soluble leptin receptor (Ob-Re) in circulation (Sinha et al., 1996; Brabant et al., 2000; Mooradian et al., 2000a). The methods used for quantifying leptin binding include ^{125}I–labelled recombinant leptin and gel-exclusion chromatography (Sinha et al., 1996; Houseknecht et al., 1996), spun column assay (Eichler et al., 1999), acid-ethanol precipitation (Mooradian et al., 2000a), RIA using polyclonal antisera against the N-terminal (to detect bound-form of leptin) and C-terminal (free form of leptin) fragments

(Lewandowski et al., 1999; Brabant et al., 2000; Brabant et al., 2002), high-pressure liquid chromatography followed by RIA of total leptin in the fractions (Landt, 2000; Quinton et al., 1999), and ligand mediated immunofunctional assay for specifically quantifying Ob-Re (Wu et al., 2002). Accordingly, depending on the method used, the molecular masses (kDa) of the leptin binding components reported were 85 (Quinton et al., 1999), 100-200 (Lewandowski et al., 1999), 176-240 (Houseknecht et al., 1996), 200-670 (Lammert et al., 2001), 280 (Sinha et al., 1996), 340 (Wu et al., 2002) and 450 (Diamond et al., 1997). Regardless of the method used, in a majority of these studies, an inverse relationship between bound leptin and fat reserves has been reported with the proportion of free leptin increasing with obesity. It is postulated that free leptin in humans reflects body fat mass and may signal the onset of puberty, whereas bound leptin may serve as a marker of resting energy expenditure (Quinton et al., 1999; Brabant et al., 2000).

The kinetics of leptin concentrations in humans and rodents has been documented. Using arterio-venous differences the half–life of leptin in humans was estimated to be 25 min (Klein et al., 1996). By measuring the decay of circulating label after injection of labelled leptin, the half-lives of leptin in normal mice, *ob/ob* mice, zucker rats, and rhesus monkeys have been estimated to be 3 h, 1.5 h, 5.5-6.9 min, and 1.5 h respectively (Klein et al., 1996; Ahima et al., 1996; Vila et al., 1998). The kidney plays a major role in the clearance of leptin from circulation. In rodents and humans, the kidney extracts nearly 25% and 80% of the circulating leptin, respectively, with nearly 97% of the extracted leptin being metabolized by the kidney and very little secreted in the urine (Meyer et al., 1997; Jensen et al., 1999).

1.3.2. *Leptin in circulation of ruminants*

1.3.2.1. *Circulating leptin concentrations determined using a 'multi-species' RIA kit*

Early studies (Chilliard et al., 1998; Minton et al., 1998) for determining circulating leptin concentrations in ruminants used a commercial "multispecies" RIA kit from Linco Research Inc. (St. Louis, MO, USA). This assay is based on antiserum raised against recombinant human leptin and utilizes human leptin standards and label. Using

this assay system we (Chelikani et al., 2000) and others (Chilliard et al., 2001, Minton et al., 1998; Gabai et al., 2002; Maciel et al., 2001; Kawakita et al., 2001; Tokuda et al., 2000) have shown that serial dilutions of bovine or sheep plasma produced curves parallel to the human leptin standard curve. However, the recoveries of human leptin added to bovine plasma ranged from 76% (Chelikani et al., 2000) to 97% (Maciel et al., 2001). With this assay system, factors regulating ciculating leptin concentrations were examined by different research groups. Some of the major findings using this assay system were that plasma leptin concentrations 1) correlated positively with carcass fatness in cattle (Minton et al., 1998; Wegner et al., 2001), 2) increased with age in both heifers and steers, with the increase being greater in heifers than in steers (Tokuda et al., 2001), 3) increased with age until 3 months after weaning in sheep (Tokuda et al., 2000), 4) exhibited a pulsatile pattern and diurnal variation in cattle (Kawakita et al., 2001), 5) decreased with feed restriction of cattle (Sansinanea et al., 2001; Chilliard et al., 2001), 6) decreased with infusion of isoproterenol in cattle (Chilliard et al., 2001), 7) increased with feeding of propionibacteria to postpartum dairy cows (Francisco et al., 2002), and 8) were unaffected by administration of dexamethasone to dairy cattle (Maciel et al., 2001).

1.3.2.2. *Circulating leptin concentrations determined using ruminant specific leptin assays*

The first reports on ruminant specific assays for quantifying circulating leptin concentrations were published in tandem in the Journal of Endocrinology between May and September 2000 (Delavaud et al., 2000; Kauter et al., 2000; Blache et al., 2000a; Ehrhardt et al., 2000) followed later by two other reports in the same journal (Marie et al., 2001; Thomas et al., 2001). The time lag between the development of monogastric and ruminant leptin assays is believed to be due to the low immunogenicity of ruminant leptin as a result of which succesful antisera could only be raised by either immunizing rabbits or birds with high doses of ruminant leptin for several months (Chilliard et al., 2001). Of these reports, two were on development of ruminant specfic ELISAs and the others on RIAs.

The ruminant specific leptin assays utilised either bovine (Blache et al., 2000a; Kauter et al., 2000; Ehrhardrt et al., 2000) or ovine (Delavaud et al., 2000; Marie et al., 2001; Thomas et al., 2001) recombinant leptin for preparation of standards and tracer (Table 1.1). The primary antisera were raised either in rabbits (Delavaud et al., 2000; Ehrhardt et al., 2000; Thomas et al., 2001), emu (Blache et al., 2000a), or chicken (Kauter et al., 2000; Marie et al., 2001). Interestingly, antisera raised against either N-terminal (6-14 amino acids) or C-terminal (124-135) amino acids of bovine leptin showed very little cross-reactivity against the intact molecule (Kauter et al., 2000). As the sheep and bovine leptins differ by only 2 amino acids, in general, the antisera crossreact with both forms and have been validated for both species. However, there appears to be some (Kauter et al., 2000) or no crossreactivity (Delavaud et al., 2000; Ehrhardt et al., 2000) of the antisera with human leptin. Thus, although the absolute leptin values determined by the ruminant RIAs were strongly correlated with the values obtained using the 'multispecies' kit, the kit understimated the true values and the ruminant RIAs were found to be more sensitive (Delavaud et al., 2000; Ehrhardt et al., 2000; Blache et al., 2000a). In general circulating leptin concentrations measured in adult cattle range from 3 – 34 ng/ml (Delavaud et al., 2002; Ehrhardt et al., 2000). In contrast to data on the existence of leptin binding proteins in humans and rodents, both gel exclusion and high pressure liquid chromatography failed to detect leptin binding proteins in bovine serum (Garcia et al., 2002).

1.4. Regulation of leptin production

1.4.1. *Adiposity*

As mentioned above, in general, circulating concentrations of leptin reflect the levels of adipose tissue reserves in rodents (Frederich et al., 1995) humans (Frederich et al., 1995; Maffei et al., 1995) and ruminants (Delavaud et al., 2000; Ehrhardt et al., 2000). A reduction in adipose tissue accompanying weight loss leads to a decrease in leptin expression and secretion in rodents, humans (Frederich et al., 1995; Maffei et al., 1995), and cattle (Block et al., 2001; Delavaud et al., 2002). Conversely an increase in adipose tissue with weight gain increases circulating concentrations of leptin in

monogastrics (Considine, 2001) and ruminants (Ehrhardt et al., 2000; Archer et al., 2002). Some of the adipose tissue characteristics that influence leptin expression and secretion include the anatomical site, adipocyte hypertrophy and hyperplasia, and genotype.

There appear to be species differences with regard to the abundance of leptin transcript in various adipose depots. The abundance of leptin mRNA was reported to be higher in subcutaenous than in omental adipose tissue of sheep (Kumar et al., 1998) and humans (Fried et al., 2000), but in rats the abundance was lower in subcutaneous relative to omental adipose depots (Villafuerte, et al., 2000). In cattle, abundance of leptin mRNA in adipose depots, as determined by Northern blotting, was found to be in the order perirenal > abdominal > subcutaneous > intermuscular (Kim et al., 2000; Ren et al., 2002); whereas no differences in transcript abundance among various depots was reported in another study using an RNAse protection assay (Ji et al., 1998). Further, among cattle breeds, leptin mRNA expression was found to be greater in subcutaneous and perirenal depots of dairy (Holstein) compared to beef (Charolais) cattle (Ren et al., 2002). Clearly, more research is needed to study the developmental changes in leptin expression among various adipose depots of cattle using sensitive techniques to address some of the discrepancies among studies.

Leptin expression and secretion is also dependent on the size of adipocytes within a depot. In women, leptin mRNA abundance is greater in larger than in smaller adipocytes and leptin secretion per fat cell is nearly 3 -7 fold greater in obese than in lean subjects (Lonnqvist et al., 1997; Van Harmelen et al., 1998). In addition to hypertrophy, adipocyte hyperplasia also contributes to the hyperleptinemia seen in human obesity (Couillard et al., 2000). In cattle, as in humans, the size of subcutanous adipose cells contributes significantly to the variation in plasma leptin concentrations (Delavaud et al., 2002).

A dual regulation of leptin secretion from adipose depots has been proposed (Levy et al., 2000). Spontaneous leptin secretion, which is independent of intracellular calcium fllux, is dependent on the amount of hormone synthesized and varies with size of and amount of triglycerides stored in an adipocyte. Thus, spontaneous or basal secretion

is responsible for the increased circulating leptin accompanying increased adiposity. Superimposed on this basal secretion, is an acute regulation of leptin secretion that is dependent on intracellular energy metabolism and calcium flux. The existence of such a dual regulatory mechanism of leptin secretion has also been proposed for sheep (Marie et al., 2001).

Within rat adipocytes nearly 60-70% of leptin mRNA was found to be associated with endoplasmic reticulum and the remaining 30-40% of the message was found in the cytpoplasm in the form of 80S mRNP particles (Roh et al., 2003). Initially, staining for leptin protein was found to be localized to endoplasmic reticulum and not on Golgi apparatus or secretory vesicles which led to the hypothesis that leptin was rapidly secreted without intracellular retention (Barr et al., 1997). However, recent studies support the view that leptin exists in preformed regulatable pools within adipocytes. For example, treatment of adipocytes with brefeldin to inhibit post-Golgi transport was found to inhibit leptin secretion (Kirchgessner et al., 1997), leptin containing compartment has been localized to low density secretory vesicles in rat adipocytes (Roh et al., 2000; 2001), and the existence of leptin stores in adipose tissue from obese humans has been demonstrated (Russell et al., 2001). Further, the amount of leptin secreted is proportional to the amount of preformed leptin found in adipocytes of obese individuals indicating that modulation of circulating leptin concentrations in obese humans is achieved through regulation of leptin both at the level of synthesis as well as secretion (Russell et al., 2001). However, details on the targetting of leptin to these vesicular compartments and the secretory mechanisms involved are not completely known.

1.4.2. *Gender*

Sexual dimorphism in circulating leptin concentrations has been documented in a number of species. In general, leptin levels are greater in the females compared to the males of a species including humans, monkeys, rodents, and sheep (Wauters et al., 2000; Blache et al., 2000a; Ehrhardt et al., 2000). The gender differences in humans are believed to be due to relatively greater subcutaneous adipose depots and leptin production per fat cell in women compared to men (Couillard et al., 2000; Montague et al., 1997b).

In addition to anatomical differences, reproductive hormones also influence leptin production in humans. A stimulatory effect of estradiol and an inhibitory effects of androgens are believed to be partly responsible for the gender differences (Wauters et al., 2000). The role of leptin in reproductive function is discussed later.

1.4.3. *Diurnal rhythm and pulsatility*

A diurnal rhythm in circulating leptin concentrations has been described in young women (Taylor et al., 1999; Matkovic et al., 1997), young men (Boden et al., 1997; Saad et al., 1998), and in both lean and obese adults (Saad et al., 1998; Sinha et al., 1996). Typically, peak leptin concentrations occur btween 2400 and 0400 h with nadirs between 1100 and 2000 h (Sinha et al., 1996). This inherent rhythm is entrained to meal timing such that shifting the meal timing by 6-7 h also shifts the peak and nadir leptin concentrations by a similar amount of time without a major shift in the noctural cortisol peak (Schoeller et al., 1997). Unlike humans, the nocturnal peak in leptin concentrations in rodents occurs at the time of feeding (Ahren, 2000). In both rodents and humans the diurnal changes in leptin concentrations were strongly associated with changes in glucose and insulin concentrations (Ahren et al., 2000; Coleman and Herrmann, 1999).

Unlike the rapid periprandial fluctuations in glucose and insulin observed in monogastrics, a more constant nutrient flow from the rumen ensures a relatively more stable glucose and insulin profile in ruminants fed a total mixed ration (TMR). Therefore, if the diurnal excursions in leptin concentrations are entrained to meal timing and associated changes in glucose and insulin, it is not surprising that no diurnal rhythm was detectable in circulating leptin concentrations in sheep (Blache et al., 2000a; Marie et al., 2001; Daniel et al., 2002) or in dairy cows during the postpartum period (Block et al., 2001). However, postprandial increases in circulating leptin concentrations have been reported in both sheep (Marie et al., 2001) and cattle (Delavaud et al., 2002). Further, using a 'multispecies' RIA kit, a diurnal rhythm with peaks between 1500 and 1600 h and nadirs at midnight were detected in Holstein steers (Kawakita et al., 2001).

Another characteristic of leptin secretion is the pulsatility observed in monogastric species. In lean and obese humans, the frequency of leptin pulses reported

have varied from 3-4 pulses/24 h (Licino et al., 1998) to 4-5 pulses/12 h (Koutkia et al., 2003). Further, the amplitude and mean concentrations of leptin pulses were shown to be directly proportional to the fat mass, whereas the pulse frequency is independent of adipose reserves (Koutkia et al., 2003). In contrast to these studies, a clear pulsatile secretion of leptin is not present in sheep, rather the secretory events were characterised as 'episodic' in nature (Blache et al., 2000a; Daniel et al., 2002). The frequency of these 'episodes' within a 24 h period in fat and thin ewes was reported to be approximately 12 and 11 in the fed state, and 12 and 6 in the fasted state, respectively (Daniel et al., 2002). Using a 'multispecies' leptin RIA kit (described earlier), Kawakita et al. (2001) were able to detect 15 pulses/24 h in 10-month old Holstein steers, and these pulses were found to be positively correlated with rumination.

1.4.4. *Fasting and undernutrition*

Short-term fasting results in a rapid decrease in circulating concentrations of leptin in rodents (Dallman et al., 1999), humans (Jeon et al., 2003; Horowitz et al., 2001; Bergendahl et al., 2000; Boden et al., 1996), sheep (Marie et al., 2001) and beef cattle (Amstalden et al., 2000). The reduction in leptin is much greater than the changes in body weight. For instance, in humans, with a 4% weight loss on fasting, plasma leptin concentrations were decreased by 54 – 76% (Boden et al., 1996; Weigle et al., 1997; Grinspoon et al., 1997). On refeeding, circulating leptin concentrations respond equally rapidly reaching basal/control levels within 12 h in humans (Weigle et al., 1997).

Because the *ob/ob* mice have similar metabolic and hormonal characteristics as those observed during starvation, the leptin deficiency in these mice is believed to represent a state of continuous starvation. Based on this logic, Ahima et al. (1996) found that preventing the fasting-induced reduction in leptin in these mice, by daily administration of exogenous leptin, prevented the fall in thyroid hormone, testosterone, luteinizing hormone, the rise in coticosterone, and shortened the estrous cycles (Ahima et al., 1996). Using a similar model, the importance of fasting-induced hypoleptinemia in mediating somatotropic, thyroid, reproductive and immune alterations has also been demonstrated in rats, non-human primates (Ahima and Flier, 2000), men (Chan et al.,

2003), and sheep (Henry et al., 1999). Therefore, it was proposed that an important role of leptin is to function as a signal for adaptation to fasting rather than signalling energy surplus (Ahima et al., 1996; Ahima and Flier, 2000).

The changes in leptin concentrations with fasting are influenced by the amount of body fat reserves. In humans, the fasting-induced reduction in plasma concentrations of leptin, insulin, and glucose, and elevation in NEFA, were reported to be blunted in obese compared to lean subjects (Horowitz et al., 2001; Landt et al., 2001; Klein et al., 2000). This reduction in leptin appears to be due to a reduction in production rather than to changes in clearance (Klein et al., 2000), and a reduction in leptin pulse amplitude rather than a change in pulse frequency (Bergendahl et al., 2000). Similar to changes in total leptin concentrations, the reduction in both bound and free leptin concentrations is also blunted in obese compared to lean individuals. Landt et al. (2001) reported that the reduction in bound and free leptin concentrations were 51% and 12% in lean individuals, and 12% and 21% in obese subjects (Landt et al., 2001). Although not discussed in the paper, the ratio of free to bound leptin seems to have remained relatively constant with fasting of obese subjects whereas this ratio was reduced with fasting of lean individuals. This shift from free to bound leptin with fasting might be an adaptive mechanism of lean individuals to reduce the bioavailability of leptin to the central nervous system.

The mechanism by which fasting downregulates leptin expression or release are not completely understood. In monogastrics, the fasting-induced reduction in leptin concentrations is believed to be due to increased sympathetic activity, increased ketones and free fatty acids, and/or due to a reduction in insulin and glucose concentrations (Coleman and Herman, 1999; Fried et al., 2001). Several lines of evidence indicate that alterations in glucose metabolism may be responsible for the fasting-induced changes in leptin production. Maintenance of euglycemia prevents the fasting-induced reduction in circulating leptin concentrations suggesting a direct role for insulin or glucose as stimulatory signals for leptin secretion (Kolaczynski et al., 1996a; Boden et al., 1996; Sonnenberg et al., 2001). Regulation of leptin secretion by glucose metabolism in isolated adipocytes has been documented (Mueller et al., 1998). The fasting-induced decline in glucose production and uptake is blunted in obese women in parallel to a

blunted leptin response (Horowitz et al., 2001). Collectively, the above mentioned studies indicate that the metabolism of glucose within adipocytes, in addition to the stimulatory effects of insulin, are responsible for the stimulatory effects of hyperglycemia and hyperinsulinemia on leptin secretion.

Apart from the attenuation of the stimulatory effects of glucose and insulin on leptin secretion under fasting conditions, an inhibitory effect on leptin secretion caused by increased nonesterified fatty acids (NEFA) has also been well documented (Arai et al., 2002; Rentsch and Chiesi, 1996). Although an inverse relationship between fasting-induced elevation in serum β-hydroxybutyrate (BHBA) and leptin concentrations has been reported, infusion of BHBA to induce hyperketonemia failed to influence serum leptin concentrations suggesting that ketone bodies may not be directly involved in the regulation of leptin release (Kolaczynski et al., 1996a).

Different experimental models including drugs that block β-adrenoreceptors, inhibitors of catecholamine synthesis, and subjects with impaired sympathetic activity, provide support to the theory that increased sympathetic activity observed with fasting inhibits leptin expression and secretion. Administration of β_1/β_2 (propanolol) or β_3 (SR 59230A) antagonists were found to prevent the reduction in leptin expression and release with fasting (Rayner and Trayhurn, 2001). Inhibition of catecholamine synthesis with α-methyl-*p*-tyrosine was reported to reverse the fasting-induced inhibition of leptin secretion in lean mice (Rayner et al., 1998). Finally, men with sympathetic dysfunction associated with injury to the spinal cord are reported to have delayed leptin response to fasting (Jeon et al., 2003). Clearly, the sympathetic nervous system plays a critical role in mediating the fasting-induced inhibition of leptin secretion.

Compared to the abundance of data on the effects of fasting on leptin expression and release in monogastrics, the adaptation of the leptin system to acute feed deprivation has received little attention in ruminants. Short-term fasting has been shown to reduce leptin gene expression (Tsuchiya et al., 1998; Amstalden et al., 2000) and circulating leptin concentrations in prepubertal beef heifers (Amstalden et al., 2000) and ovariectomized, estradiol–implanted beef cows (Amstalden et al., 2002). Similarly,

leptin gene expression (Kumar et al., 1998) and circulating leptin concentrations (Marie et al., 2001; Nagatani et al., 2000; Daniel et al., 2002) were reported to be reduced with fasting in sheep. This fasting-induced reduction in plasma leptin concentrations in sheep is influenced by photoperiod and body fat reserves. Leptin concentrations were reduced to basal levels by 26 h and 33 h of fasting in ewes exposed to short and long-day length, respectively (Marie et al., 2001). Although, leptin concentrations were reduced with fasting of both fat and thin ewes, the peak frequency was unaltered in fat ewes but was reduced with fasting of thin ewes (Daniel et al., 2002). However, none of these studies reported the time-course of changes in leptin concentrations with refeeding. Further, the effects of fasting and refeeding on temporal changes in circulating leptin concentrations in dairy cattle have not been reported to date.

In addition to the acute changes in leptin that occur within hours of fasting, undernutrition over a period of days also has a significant impact on circulating concentrations of leptin. For example, plasma leptin concentrations were reduced by 76% in men who lost 21% of their weight over a 95-d period (Weigle et al., 1997). Similarly, lean adults on a weight reducing diet lost 4% of their body weight with a 36% reduction in circulating leptin concentrations (Dubuc et al., 1998). Reduction in circulating leptin with moderate energy deficit was found to be correlated with increased hunger sensations in women (Keim et al., 1998). On the other hand, overfeeding men to increase weight by 10% (~4% fat gain) was associated with nearly a three-fold increase in cicrulating leptin concentrations (Kolaczynski et al., 1996b). Similar to these human studies, leptin concentrations in ruminants also respond rapidly to long-term feed restriction and realimentation. In fact, in adult ewes, body fatness and feeding level contributed to 35 and 17% of the variation in plasma leptin concentrations, respectively (Delavaud et al., 2000). Lambs fed to grow at 0.4 kg/d had 51% greater plasma leptin levels than those fed for a growth rate of 0.3 kg/d (Ehrhardt et al., 2000); plasma leptin concentrations were doubled within 5 d of switching to a high plane of nutrition in adult rams (Blache et al., 2000a); circulating leptin concentrations were increased within 2 (Thomas et al., 2001) to 3 days (Archer et al., 2002) of changing from a moderate to a high level of intake. Similarly, plasma leptin concentrations were found to be rapidly

increased in dairy cows fed to excess of requirements during the dry period (Holtenius et al., 2003). Thus, across species, the rapid changes in leptin in response to both acute and medium term fasting or undernutrition are disproportionate to accompanying changes in body weight or fat reserves. The reduction in leptin concentrations with fasting or undernutrition, not only informs the brain of the reduced energy intake but also of a reduction in adipose tissue reserves. This has led to the hypothesis that "leptin acts most potently at low levels of energy sufficiency and may be particularly important in signalling across the transition between energy sufficiency and insufficiency" (Prentice et al., 2002).

1.4.5. *Regulation by macronutrients*

1.4.5.1. *Dietary fats*

1.4.5.1.1. *Rodents and humans*

The effects of dietary fat on leptin expression and release are quite complex and vary depending on a number of factors including the duration of feeding, the type and amount of fat fed, and the fatty acid composition.

The regulation of plasma leptin concentrations by dietary fat was first addressed by Frederich et al. (1995). In this study feeding a diet rich in saturated fat (~41% of energy from fat) for 12 weeks was found to increase plasma leptin concentrations by over four-fold in male, and two-fold in female mice. As the caloric intake of these mice was not reduced, despite the hyperleptinemia, it was suggested that increased intake of fat changes the 'set point' for body weight by interfering with leptin action. The stimulatory effects of saturated fat diets on leptin secretion in rodents have since been reported by other researchers (Ahren et al., 1997; Lin et al., 1998). The insensitivity to hyperleptinemia was further elaborated by Lin et al. (2000) who described the temporal changes in the development of leptin resistance after long term feeding of a high fat diet to mice: 1) an early stage (3 weeks) with high sensitivity to exogenous leptin; 2) a reduced food intake stage (8 weeks) during which mice had increased leptin production but were still sensitive to leptin; and 3) an increased food intake stage (19 weeks) during which the mice exhibited leptin resistance. In accordance with this, feeding diets

enriched in saturated fat (~45% of total energy from fat) to mice for 9 weeks did not change Ob-Rb gene expression in the hypothalamus (Sahu et al., 2002), but by 19 weeks feeding the STAT 3 DNA binding activity was reduced in the hypothalamus (El-Haschimi et al., 2000). However, feeding saturated fat was found to result in a 11-fold increase Ob-Ra expression at the blood brain barrier (Boado et al., 1998).

The influence of dietary fatty acid composition on leptin secretion was first examined by Cha and Jones (1998). In this study, rats fed safflower oil or fish oil had 60% higher plasma leptin concentrations compared to those fed beef tallow without any treatment differences in body weight. Similarly, rats fed fish oil diets had nearly 75% greater plasma leptin concentrations compared to controls without any changes in body weight (Peyron-Caso et al., 2002). However, other reports found that feeding n-3 polyunsaturated fatty acids reduced leptin gene expression (Reseland et al., 2001) and plasma leptin concentrations (Hun et al., 1999) in rodents in association with a reduction in body weight.

Human studies showed that fat supplementation either had no effect, increased or decreased circulating leptin concentrations. Circulating leptin concentrations were unchanged in human subjects consuming either 60% (Schrauwen et al., 1997) or 31% of total energy as fat (Havel et al., 1996). In contrast to these studies which considered only morning blood samples, in subjects consuming either a low-fat high-carbohydrate diet the area under the curve for the leptin profile was either reduced (Havel et al., 1999) or not affected (Weigle et al., 2003), the amplitude of the 24-h leptin profile was increased (Havel et al., 1999; Weigle et al., 2003) and the amplitude was negatively correlated with weight loss suggesting an increased sensitivity of the CNS to leptin (Weigle et al., 2003). Infusion of a triglyceride emulsion (Intralipid®) either through oral or intravenous routes reduced plasma leptin concentrations in women (Evans et al., 2001b). In contrast, intravenous infusion of Intralipid® was found to increase leptin mRNA expression in adipose tissue of both humans (Nisoli et al., 2000) and rodents (Wang et al., 1998), but had no effect on circulating leptin concentrations in humans (Stingl et al., 2002). Recently, Kratz et al. (2002) reported that circulating leptin concentrations were

decreased in women on a rapeseed oil (enriched in 18:3n-3) but increased in men on the same diet. In the same study, olive oil (high in 18:1) or sunflower oil (high in 18:2) had no effect on leptin concentrations in circulation, thereby indicating a specific stimulatory effect of 18:3n-3 enriched diets on leptin secretion. In addition to the degree of unsaturation and chain length, specific fatty acid isomers are also involved in the regulation of leptin secretion. For example, feeding a mixture of conjugated linoleic acid (CLA) isomers for 8 weeks was found to reduce circulating leptin concentrations in women without any changes in body fat (Medina et al., 2000). A similar response was also observed in rats (Yamasaki et al., 2003). Further, an inverse relationship between *trans*-10 *cis*-12 CLA and leptin concentrations in circulation was observed in humans (Belury et al., 2003).

1.4.5.1.2. *Ruminants*

Because dry matter intake is insufficient to maintain a positive energy state in ruminants during early lactation, energy intake can be improved by feeding high concentrate diets or supplementing the diet with fat. However, feeding high levels of fat was shown to depress feed intake and increase the loss of body weight and body condition, especially before peak lactation in dairy cows (Chilliard, 1993). It is speculated that multiple regulators are involved in mediating the fat-induced depression in dry matter intake depending on the level and the type of added fat in the diet. The depression in feed intake is more pronounced with feeding unsaturated fat sources compared to saturated fat supplements (Allen, 2000). Though the identity of the satiety factors mediating the fat-induced depression in feed intake is not completely known, cholecystokinin (CCK), pancreatic polypeptide, and glucagon-like peptide 1 are potential candidates (Choi et al., 2000; Suominen et al., 1998). In a recent study, abomasal infusion of canola oil for four hours significantly increased CCK mRNA abundance in the duodenum and also increased plasma CCK concentration (Suominen et al., 1998). Although feed intake was unaffected, possibly due to the relatively short duration of infusion, this finding is evidence that gastrointestinal cells in the gut are highly sensitive to alterations in macronutrients supplied to the animal. In recent studies, plasma leptin

cocentrations, were reported to be unaffected by CLA supplementation to dairy cows (Baumgard et al., 2002) or by feeding diets rich in 18:2 to beef heifers (Garcia et al., 2003). In contrast, feeding a 'rumen protected' fat to fat-tailed sheep for 15 d was found to increase plasma leptin concentrations (Yildiz et al., 2003) together with increases in body condition score (BCS: a proxy to body fat reserves). However, in cattle, the relative importance of gut- versus adipose tissue-derived satiety signals such as leptin in regulation of feed intake, the time frame over which these signals operate, the response of these signals to the type of unsaturated fatty acids reaching the intestine, and the interaction of these signals with the body condition of the animal, are still unknown.

1.4.5.2. *Carbohydrates*

1.4.5.2.1. *Rodents and humans*

The importance of carbohydrates in the regulation of leptin secretion has been well documented in rodents and humans. As mentioned earlier, maintenance of hyperglycemia prevented the fasting-induced reduction in plasma leptin concentrations suggesting a direct effect of glucose metabolism in the regulation of leptin secretion (Boden et al., 1996). In the short-term, circulating leptin concentrations were found to be strongly correlated with changes in dietary carbohydrate intake in humans (Jenkins et al., 1997). Human subjects consuming a low-fat high-carbohydrate meal, which caused large increases in insulin and glucose, had greater 24 h leptin concentrations than subjects on a high-fat low-carbohydrate diet (Havel et al., 1999). Similarly, in both men and women, a carbohydrate meal was found to generate a greater postprandial leptin response than an isoenergetic fat meal within 4-5 h after food ingestion, and this response was found to be correlated to the postprandial insulin changes (Romon et al., 1999). Whereas glucose and sucrose feeding elicited a hyperleptinemic response in rodents (Mooradian et al., 2000b; Suga et al., 2000), feeding fructose either increased (Mooradian et al., 2000b) did not affect (Suga et al., 2000), or reduced circulating leptin concentrations (Elliott et al., 2002).

The role of specific amino acids in the regulation of leptin secretion are not completely known. Infusion of a mixture of amino acids did not affect leptin

concentrations in humans (Drewes et al., 1997), whereas leptin production was increased 2-3 fold by leucine in isolated rat adipocytes (Roh et al., 2003), and plasma leptin concentrations were decreased by infusion of arginine to humans (Stingl et al., 2002).

1.4.5.2.2. *Ruminants*

Unlike monogastric species, in ruminants, the majority of the digestion occurs pregastrically i.e. in the rumen. Carbohydrates in ruminant diets consist of both structural (fiber – primarily from 'forages') and non-structural (sugar, starches, pectins- primarily from grain that constitute the 'concentrate' portion of the diet). Non-structural carbohydrates undergo extensive fermentation in the rumen to yield volatile fatty acids (VFA; primarily acetate, propionate (glucogenic), and butyrate) which in turn provide a majority of the total energy supply. Depending on the ratio of the concentrate to forage in the diet, type of forage or grain used, the extent of processing of the grain or forage, and other factors, the amount of starch or glucose available to the intestine can be increased substantially. The relative importance of these factors in the regulation of leptin secretion in cattle are just being addressed.

In a recent study by Reist et al. (2003), feeding a high concentrate diet (at 50% of total intake) to dairy cows for 10 weeks postpartum was found to significantly increase plasma concentrations of leptin, insulin, glucose and IGF-1 concentrations, and also increase body weight and BCS, when compared to cows on a low concentrate diet (concentrate fed at 30% of total intake). A stimulatory effect of propionate on leptin secretion maybe involved because feeding propionibacteria was found to increase plasma leptin concentrations postpartum (Francisco et al., 2002), and cows fed a 'glucogenic' feed had greater plasma leptin concentrations relative to cows not receiving the feed (Kokkonen et al., 2002). Similarly, steers receiving a maize silage tended to have higher plasma leptin concentrations compared to steers receving a grass silage (Faulconier et al., 2002). In addition to the amount and type of forage or concentrate fed, the environmental temperature and the method of feeding also appear to influence circulating leptin concentrations. Plasma leptin concentrations were increased in dairy cows housed under warm (+10^{0}C) than those in cold (+2^{0}C) housing conditions, and the increase was greater

with feeding as a total mixed ration (TMR) than feeding the concentrates and forages seperately (Kokkonen et al., 2002). Similarly, in sheep, a high carbohydrate (barley-rich) diet was found to increase plasma leptin concentrations along with an improvement in BCS (Yildiz et al., 2003). In contrast to these studies, a single bolus infusion of glucose did not alter plasm leptin concentrations in sheep (Kauter et al., 2000). Controlled studies with sustained infusions of VFA or glucose are needed to further understand the role of glucogenic precusors or glucose in the regulation of leptin secretion in cattle

1.4.5.3. *Mechanisms of action of macronutrients on leptin expression and release*

1.4.5.3.1. *Role of Insulin*

Several lines of evidence indicate the effects of macronutrients, especially carbohydrate-rich diets, on leptin expression and release are mediated by insulin. A direct stimulatory effect of insulin on leptin secretion has been well-documented in a number of studies in rodents and humans (Kieffer et al., 2000; Coleman and Herrmann, 1999; Considine, 2001). In general, either 4-6 h of supraphysiological hyperinsulinemia (Utriainen et al., 1996) or 24-72 h of physiological hyperinsulinemia (Saad et al., 1998; Boden et al., 1997) are required to increase circulating leptin concentrations in humans. The increase in circulating leptin concentrations caused by carbohydrate-rich diets are often preceded by, or coincide with, the increase in insulin concentrations (Romon et al., 1999; Havel et al.., 1999; Weigle et al., 2003).

Rather than insulin *per se*, insulin-mediated glucose metabolism appears to be necessary for stimulation of leptin secretion. In humans, using the blood glucose–clamp technique with exogenous administration of insulin, serum leptin concentrations were found to be increased by approximately 20% with low-insulin-hypoglycemic clamp, 33% with high-insulin-hypoglycemic clamp, 39% with low-insulin-euglycemic clamp, and 60% with high-insulin-euglycemic clamp. The key regulatory steps in insulin-mediated glucose metabolism were identified by Mueller et al. (1998) using rat adipocytes. In this study, preventing glucose uptake and glucokinase-induced phosphorylation by 2-deoxy-D-glucose, or inhibition of glycolysis with NaF, was found to inhibit leptin expression and secretion even in the presence of high concentrations of insulin. Further, oxidative

metabolism of glucose beyond pyruvate, with generation of ATP's and a rise in intracellular calcium, appear to be necessary for glucose-mediated stimulation of leptin secretion (Levy et al., 2000).

Recently, Roh et al. (2003) proposed that insulin regulates leptin expression at the level of translation via the mammalian target of rapamycin (mTOR) pathway. The two major components of GLUT4 vesicles in fat and skeletal muscle cells are GLUT4 and an insulin-responsive amino peptidase (IRAP). Upon insulin-stimulated translocation of these vesicles to the cell surface, IRAP mediates a substrate-dependent release of free leucine which in turn activates mTOR. Through complex interaction with elongation factors, mTOR stimulates translation of leptin mRNA. Thus, for the first time, a stimulatory effect of an amino acid, leucine, on leptin expression has been linked to insulin regulation of leptin expression.

1.4.5.3.2. *Role of hexosamine biosynthetic pathway*

Wang et al. (1998) proposed that the flux of carbon through the hexosamine biosynthetic pathway plays an important role in the mediating the stimulatory effect of glucose and lipid on leptin expression and release from adipose tissue and muscle. Approximately 1-3% of glucose uptake into the cell enters the hexosamine pathway. Following phosphorylation of glucose to glucose-6-phosphate and then isomerization to fructose-6-phosphate, the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT) catalyzes the transfer of the amido group from glutamine to fructose-6-phosphate to form glucosamine-6-phosphate. Several other non rate-limiting steps yield UDP-*N*-acetylglucosamine, the final end-product of this pathway. Increased availability of free fatty acids, as with lipid infusions, generates increased levels of fatty-acylCoA, which inhibits glycolysis, thereby resulting in accumulation of fructose-6-phosphate and it's subsequent conversion to UDP-*N*-acetylglucosamine, the end product of the hexosamine pathway. The covalent transfer of N-acetylglucosamine from UDP-*N*-acetylglucosamine to serine/threonine residues of the transcription factor Sp1 protects Sp1 from degradation, allows it to bind to GC rich boxes on the leptin promoter, and activate leptin expression (Zhang et al., 2002). In rats, infusion of glucose, glucosamine,

uridine, or lipid was shown to increase UDP-*N*-acetylglucosamine concentrations in adipose and muscle tissue with subsequent rapid increases in leptin expression and release (Wang et al., 1998). In humans, UDP-*N*-acetylglucosamine content of adipose tissue was found to be elevated nearly three-fold in obese compared to lean subjects, UDP-*N*-acetylglucosamine was found to stimulate leptin secretion from adipocytes in culture (Considine et al., 2000). Although, glucose and gluosamine seem to act through the hexosamine pathway, a direct effect of both substrates in stimulating leptin promoter activity independent of Sp1 has also been reported (Zhang et al., 2002).

1.4.5.4. *Regulation of leptin production by other factors*

As mentoned earlier, the leptin gene promoter of humans, rodents and cattle is highly conserved, and contains a TATA box, a C/EBP binding sequence and three GC-rich boxes for binding Sp1. The human leptin gene promoter also has a glucocorticoid response element (GRE) located between –1283 to –1286 bp and cAMP response elements (CREB) at –1384 to 11380 bp (Considine, 2001). In addition *cis* elements for glucocorticoid and peroxisome proliferator activated receptor γ (PPARγ) located between –55 and +31 bp, and between –65 and +9 bp, respectively (Considine, 2001).

Although, the transcriptional regulation of leptin expression by glucocorticoids is not completely understood, there is substantial *in vitro* and *in vivo* evidence indicating that glucocorticoids stimulate leptin expression and release in both rodent and human models (Fried et al., 2000; Dagogo-Jack, 1999; Considine, 2001). However, the paradoxical increase in circulating cortisol concentrations with fasting or energy restriction, concomitant with reduction in leptin concentrations, suggest that endogenous glucocorticoids likely have a minor modulatory role in the regulation of leptin in humans (Dubuc et al., 1998). Administration of dexamethasone did not affect plasma leptin concentrations in dairy cows (Maciel et al., 2001) whereas dexamethasone increased leptin mRNA abundance in adipose tissue explants from beef cattle (Houseknecht et al., 2000). PPARγ agonists are believed to supress leptin gene expression and release either directly or by antagonizing the binding of C/EBPα to the leptin gene promoter, whereas

β-agonists and cAMP inhibit leptin expression likely through interaction with CREB elements (Considine, 2001). Cytokines such as tumor necrosis factor α (TNF α) were reported to stimulate leptin secretion in the short-term and inhibit leptin secretion in the long term (Considine, 2001). Administration of pancreatic polypeptide was found to reduce leptin expression in adipose tissue (Asakawa et al., 2003), whereas administration of Ghrelin (an orexigenic signal from stomach) did not have a direct effect on plasma leptin concentrations in rodents (Tolle et al., 2001).

The effects of growth hormone (GH) and insulin-like growth factor (IGF-1) on leptin expression and release are relatively complex. Administration of GH was shown to decrease plasma leptin concentration in children independent of changes in body mass index (Elimam et al., 1999) and decrease leptin gene expression in adipose tissue of Zucker rats (Isozaki et al., 1999). Combined administration of GH and IGF-1 for 3 days was found to increase plasma leptin concentrations whereas chronic (3 months) administration of GH to GH-deficient adults was found to decrease body fat stores and circulating leptin concentrations (Dagogo-Jack, 1999). A single bolus injection of GH to adult humans was found to increase plasma leptin concentrations within 18-48 h followed by a nadir between 48-120 h (Lissett et al., 2000). In adipose tissue explants from cattle, GH was found to attenuate the stimulatory effects of insulin and dexamethasone on leptin expression whereas *in vivo* GH administration to growing steers for 3 days increased leptin mRNA abundance in adipose tissue (Houseknecht et al., 2000). Further IGF-1 expression in adipose tissue was strongly and positively correlated with changes in leptin expression (Houseknecht et al., 2000). Transgenic sheep with enhanced GH production, and low body fat reserves, were found to have low circulating concentrations of leptin (Kadokawa et al., 2003a). An inverse relationship between high GH concentrations and low leptin concentrations during the postpartum period has also been documented in dairy cows (Block et al., 2001; Holtenius et al., 2003; Reist et al., 2002; 2003). Thus, the leptin response to GH appears to be dependent on the duration of GH treatment or the duration of elevation in circulating GH concentrations. In the short-term GH appears to

have a stimulatory effect on leptin expression and release whereas in the long-term GH is inhibitory to leptin secretion posssibly due to a reduction in body fat reserves.

1.5. Role of leptin in the regulation of food (feed) intake and puberty

Earlier studies on leptin physiology, focussed on understanding the role of the hormone in the regulation of food intake, energy balance and reproduction. However, the diversity of the tissues expressing leptin receptors, as mentioned in earlier sections, has provided evidence that the hormone is capable of regulating a range of physiological functions including immunity, stress response, bone development, angiogenesis and wound healing, carbohydrate and fat metabolism and regulation of blood pressure (Fruhbeck, 2002; Wauters et al., 2000; Harris, 2000). Despite such functional diversity of this hormone, regulation of food intake and reproduction remain as important functions. As the central theme of this dissertation is to gain an understanding of the role of leptin in mediating the effects of nutrition on feed intake and puberty, available data on the effects of exogenous leptin on food intake, neuroendocrine adaptations and puberty in monogastrics and ruminants are briefly reviewed here.

1.5.1. *Role of leptin in the regulation of food (feed) intake and neuroendocrine axis*

The most well-known function of leptin is its ability to reduce food (feed) intake in a variety of species. Peripheral or central administration of leptin reduced food intake in *ob/ob* mice, and wild type mice but not in the *db/db* mice that have a truncated receptor isoform which is incapable of mediating JAK-STAT signalling (Vaisse et al., 1996; Ghilardi et al., 1996; Halaas et al., 1995; Campfield et al., 1995; Pelleymounter et al., 1995). Hyperleptinemia induced by adenovirus-mediated leptin gene therapy also reduced food intake and body weight considerably (Chen et al., 1996). Using a similar leptin-gene therapy approach, Wang et al. (1999) report that an increase in circulating leptin concentrations within the physiological range acts directly on the hypothalamus to reduce food intake, whereas increments in leptin concentrations above the physiological range reduces adipose reserves through a combined hypothalamic effect on intake and a direct depletion of triglyceride stores in adipocytes. Peripheral administration of leptin

has also been shown to reduce appetite in humans (Hukshorn et al., 2003; Westerterp-Plantenga et al., 2001), and central administration of leptin was shown to reduce food intake in monkeys (Tang-Christensen et al., 1999). In sheep, central administration of leptin caused a profound inhibition of feed intake, especially in well-fed sheep, whereas peripheral administration had no affect. Further central administration of leptin corrects some of the alterations in hormone sceretion observed with fasting. A summary of the effects of central or peripheral administration of leptin on feed intake and changes in hormones and metabolites in farm animals is shown in Table 1.2.

Identification of the central targets of leptin action is not the focus of this dissertation; therefore, a detailed description of the neural networks through which leptin exerts its effects is beyond the scope of this review. However, a brief summary of the neural pathways affected by leptin is provided here. Lesions of the paraventicular (PVN), dorsomedial (DMH) and lateral hypothalamic (LH) nuclei cause obesity in rodents (Choi et al., 1999) and Ob-Rb has been shown to be expressed in these nuclei in sheep (Dyer et al., 1997b; Williams et al., 1999). As mentioned earlier Ob-Rb is coexpressed with NPY, POMC, MCH, CRF, and orexins in sheep (Iqbal et al., 2001) and also neurons staining postive for cocaine-amphetamine-regulated transcript (CART) in rodents (Ahima and Flier, 2000). The interactions of these neuropeptides with leptin is an exciting research area and has been extensively reviewed (Schwartz et al., 2003; Spiegelman and Flier, 2001; Ahima and Osei, 2001). There are two populations of neurons in the arcuate nucleus that are directly responsive to leptin. One set of these'first-order' neurons contain both orexigenic peptides -NPY and agouti-related transcript (AGRP), whereas the other set coexpress the anorexigenic neuropeptides POMC and CART. Leptin stimulates production of α-MSH (a product of POMC) and inhibits the release of AgRP via STAT3, whereas the inhibition of NPY by leptin occurs independent of STAT3 dependent Ob-Rb signalling (Bates et al., 2003). Binding of α-MSH to MC4 receptors on 'second order' hypothalamic targets competitively inhibits AgRP binding to the same receptor. The neurons expressing MC4 receptors include, TRH neurons in the PVN that regulate the thyroid, MCH neurons in the LH that regulate

feeding, and others. Thus, neuronal outputs from the MC4-receptor neurons are capable of influencing the thyroid, growth and reproduction through pituitary function, and behavioral and autonomic outputs such as feeding and energy expenditure (Spiegelman and Flier, 2001).

In addition to the regulation of long-term energy balance, leptin has also been shown to modulate the effects of short-term satiety signals. The gut hormone, cholecystokinin (CCK), which is secreted in reponse to nutritional stimuli has been shown to inhibit food intake in monogastrics by inhibition of gastric emptying and stimulation of vagal afferent fibers to nucleus of the solitary tract (NTS) in the hind brain (Moran, 2000). Several lines of evidence indicate a synergistic role for leptin and CCK in the regulation food intake. Leptin potentiates the satiety effects of CCK in the short-term (Emond et al., 1999; Wang et al., 2000); leptin and CCK together cause a greater reduction in food intake and body weight in the long-term than either peptide alone (Matson and Ritter, 1999; Matson et al., 2000) in rodents. The expression of leptin and Ob-Rb mRNA was detected in the gastrointestinal tract of both humans and rodents (Sobhani et al., 2000; Bado et al., 1998; Morton et al., 1998); while CCK_B receptor mRNA was detected in adipose tissue of rodents (Attoub et al., 1999). Further, leptin increases the activity of vagal afferents in synergism with CCK (Attele et al., 2002), and CCK increases mRNA and protein expression of gastric leptin (Bado et al., 1998). Although, a synergistic interaction between leptin and CCK in modulating food intake occurs in humans and rodents, the existence of such an interaction has not been documented in any farm animal species to date.

1.5.2. *Role of leptin in the regulation of puberty*

1.5.2.1 *Nutrition and puberty in cattle*

The importance of nutrition in regulating the onset of puberty is well established in both ruminants (Schillo, 1992; Sejrsen et al., 1994) and monogastrics (Foster and Nagatani , 1999). Puberty in ruminants is the result of a series of maturation events within the reproductive system that begin early in life and continue through the peripubertal period. In prepubertal heifers, the ovarian follicles grow and regress in

waves and estradiol concentrations increase with age (Evans et al., 1994). During this period, GnRH neurones in the hypothalamus are inhibited by negative feedback effects of estradiol. As puberty advances, the GnRH pulse generator becomes less sensitive to the inhibitory effects of estradiol resulting in increased LH pulse frequency, enhanced follicular maturation, and a series of events culminating in ovulation (Kinder et al., 1995).

In Holstein heifers, puberty generally occurs at 9 to 11 months of age and at an average body weight of 250 to 280 kg. However, both the age and weight at puberty can vary widely from 5 to 20 months of age, and from 200 to 300 kg body weight (Sejrsen, 1994). At puberty there is a transition from a 'reproductively quiescent' prepubertal phase to a sexually active postpubertal period. Coincident with this transition in reproductive tract development, mammary growth switches from an allometric phase in the prepubertal period to an isometric growth period postpubertally (Sejrsen, 1994). Allometric mammary growth (in which mammary parenchyma grows at ~ 3 times the general body growth), that occurs between 3 to 9 months of age, seems to be coordinated with changes in the ovaries, and is particularly sensitive to the dietary energy levels and rate of weight gain (Sejrsen et al., 2000). There is substantial evidence to suggest that energy dense diets in the prepubertal period cause excessive fat deposition in the mammary gland at the expense of secretory tissue, the milk-producing mammary parenchyma. Thus, as the primary objective of dairy heifer management is to maximize milk production potential, a number of studies focussed on the effects of nutrition on mammary development. Data on changes in body composition and the temporal changes in hormonal and metabolic signals during the pubertal transition, is relatively scarce in dairy heifers.

In most studies heifers reared on a high plane of nutrition grew rapidly, were well-conditioned, and reached puberty at a younger age although at a similar body weight. This would imply that puberty occurs when the animal attains a certain body weight or when it accumulates a certain level of fatness. Clearly, the degree of adiposity is somehow sensed by the brain, which in turn leads to a pulsatile secretion of GnRH and hence of LH. However, it is not known which metabolic and hormonal signals relay

information to the brain about the level of body reserves and thus trigger GnRH release at puberty. Possible candidates for this role include glucose, insulin, IGF-1, tyrosine (Schillo et al., 1992) and leptin (Foster and Nagatani, 1999).

1.5.2.2. *Peripubertal changes in circulating leptin concentrations and effects of leptin administration on onset of puberty*

The landmark paper by Chehab et al. (1996) reporting that leptin injections to *ob/ob* mice restored their reproductive capacity, provided the first evidence linking body fat reserves, reproduction and leptin. Later, the same group provided evidence for a direct involvement of leptin in advancing the onset of puberty (Chehab et al., 1997). Normal prepubertal female mice injected with leptin had slower weight gain than controls but experienced precocious puberty as evidenced by classical signs such as vaginal opening, oestrus and cycling (Chehab et al., 1997). In a study to determine whether leptin can advance the onset of puberty in underfed rats, Cheung et al. (1997) found that leptin treatment to underfed rats only partially restored the timing of pubertal maturation. Therefore, the authors concluded that leptin is a 'permissive factor' and acts as a 'metabolic gate' to allow sexual maturation to proceed only under adequate metabolic conditions.

Given the difficulties, and the expense involved with administration of reasonably large doses of leptin over a protracted period of time in large animals and primates, a number of studies focused on quantifying the temporal changes in circulating leptin concentrations during the peripubertal period in an attempt to identify whether there is 'threshold' level of leptin for puberty to occur, and/or if there is a prepubertal increase in circulating leptin concentrations. Leptin levels were shown to increase steadily with approaching puberty in girls (Blum et al., 1997) and mice (Chehab et al., 1997); reach peak levels around Tanner stage 2 in boys followed by a gradual decline throughout the remainder of puberty (Blum et al., 1997). Peripubertal changes in leptin were undetectable in male rhesus monkeys (Plant and Durrant, 1997), whereas in castrated male monkeys a nocturnal increase in leptin, GH and IGF-1 concentrations was found to precede LH pulses at 10-30 days before the onset of puberty (Suter et al., 2000).

Although, the exact mechanisms responsible for this sexual dimorphism in leptin concentrations are unknown, a stimulatory effect of estradiol and an inhibitory effect of testosterone on leptin expression and release might be involved (Caprio et al., 2001). As in monogastrics species, a steady prepubertal increase in circulating leptin concentrations, independent of dietary manipulation, has recently been reported in beef heifers (Garcia et al., 2002, 2003). However, it is not yet known whether puberty in dairy heifers occurs at a constant level of body fat reserves and whether circulating leptin concentrations increase with the onset of puberty.

1.5.2.2 *Effects of leptin on the hypothalamo-pituitary-gonadal axis*

Across species, short-term fasting has a profound influence on the reproductive axis, with LH secretion typically being reduced with fasting. Using this fasting-refeeding paradigm, Ahima et al. (1996) demonstrated that leptin reverses the reduction in plasma LH concentrations and the delay in ovulation with fasting. By using a similar experimental approach the ability of leptin to prevent the fasting-induced changes in LH concentrations has since been reported in other species including sheep (Nagatani et al., 2000), cattle (Amstalden et al., 2002), and monkeys (Finn et al., 1998). In addition to these effects leptin administration has also been shown to directly stimulate LH secretion in both ruminants and swine (Table 1.2). Thus, these data suggest that leptin exerts a direct affect on central networks that regulate pituitary gonadotropin secretion.

Although, the distribution of leptin receptors in various brain regions has been reported (discussed in earlier sections), the neuronal pathways by which leptin influences GnRH secretion are not completely known. In both rat and monkeys, little coexpression of Ob-Rb with GnRH neurons at both mRNA and protein levels has been reported (Cunnigham et al., 1999) making it unlikely that leptin exerts direct effects on GnRH neurons. However, Ob-Rb is strongly coexpressed with POMC and NPY containing cells, and as these neuropeptides are involved in the regulation of GnRH secretion, it is likely that leptin exerts indirect effects on GnRH through POMC and NPY regulated pathways in both monogastrics and sheep (Iqbal et al., 2001; Cunningham et al., 1999). Whereas Ob-Rb-STAT3 signalling is required for regulation of energy balance, STAT3-

independent signals triggered by Ob-Rb appear to be important in the regulation of NPY expression, and hence of fertility, growth, and glucose homeostasis in rodents (Bates et al., 2003). Nevertheless, in hypothalamic explants of rodents, low doses of leptin stimulate whereas high doses inhibit GnRH secretion (Yu et al., 1997). In contrast to the weak immunostaining of Ob-Rb with GnRH neurons, as mentioned earlier in sheep, 90% and 29% of the gonadotropes of the pars tuberalis and pars distalis coexpress Ob-Rb, respectively (Iqbal et al., 2001). Further, leptin has been shown to stimulate LH secretion from anterior pituitary explants from cows (Williams et al., 2002). Thus, leptin could potentially regulate gonadotropin secretion from the pituitary directly, apart from an indirect control on GnRH secretion.

There is substantial evidence indicating that leptin exerts direct effects on the ovary. The expression of Ob-Rb mRNA was detectable in the ovaries of rodents (Hoggard et al., 1998), humans (Karlsson et al., 1997), and dairy cattle (Silva et al., 2002). In addition, a concentration-dependent binding of ^{125}I-mouse leptin to isolated bovine granulosa and theca cells has been demonstrated (Spicer and Francisco, 1997, 1998). Although leptin is present in the follicular fluid it is controversial whether leptin is synthesized by the ovary or is extracted from serum by the ovary (Smith et al., 2002b). Regardless of its origin, several lines of *in vitro* evidence indicate that leptin inhibits steroidogenesis in theca and granulosa cells of the ovary. In the bovine ovary, high concentrations of leptin (>10 ng/ml) directly antagonizes the stimulatory effects of insulin on aromatase activity, progesterone and estrogen production by granulosa cells, and progesterone production by theca cells; however, insulin-induced proliferation of theca cells is stimulated by leptin (Spicer and Francisco, 1998, 1997). Therefore, Spicer (2001) hypothesized that moderate increases in increasing (moderate) concentrations of leptin (< 10 ng/ml) that occur during puberty or during refeeding from a nutritionally deprived state will stimulate the reproductive axis at the level of the hypothalamus or pituitary. Under conditions of undernutrition, ovarian function is primarily dependent on support from gonadotropins, insulin and/or IGF-1. When leptin concentrations are excessively high, as in obesity, leptin is inhibitory to steroid production from the ovary (Spicer, 2001).

1.6. Conclusion

Despite convincing evidence from several studies in monogastric species on the role of leptin in communicating the levels of fat reserves to the brain to regulate food intake and reproduction, the basic physiological mechanisms linking body fat reserves to feed intake and reproductive performance in dairy cattle are still unknown. Several important questions need to be addressed: (1) Under what conditions does adipose tissue signal the brain to regulate feed intake?, (2) Do long-term signals interact with short-term signals to control feed intake in cattle?, (3) What is the role of adipose tissue in controlling the onset of puberty?, and (4) What is the role of leptin in these responses? These are some of the questions that were the subject of this dissertation. An understanding of these mechanisms should provide valuable information for developing effective feeding strategies that could maximize feed intake and improve productive and reproductive performance of dairy cattle.

1.7. Research objectives

The overall objective of this research was to gain an understanding of the nutritional regulation of leptin secretion, and the possible role of leptin in the regulation of feed intake and puberty in dairy cattle. The specific objectives were:

1. To determine the tissue expression of leptin and leptin receptor mRNA in various central and peripheral tissues.
2. To determine whether the leptin response to short-term fasting is dependent on the body condition and physiological stage of the animal, and whether the leptin response is affected by partial dilution of rumen contents. Also to study the relationship of circulating leptin concentrations with insulin, insulin-like growth factor-1 (IGF-1), growth hormone (GH), glucose, and nonesterified fatty acids (NEFA) under fasting-refeeding conditions.
3. To determine the effect of feeding versus postruminal infusion of unsaturated fat on:
   (i) Feed intake, body condition, and rumen fermentation characteristics.

(ii) The expression of the cholecystokinin (CCK) and leptin genes in the duodenum, and of the leptin, acetyl-coA carboxylase and lipoprotein lipase genes in adipose tissue.

(iii) Blood concentrations of CCK, leptin, insulin, glucose, IGF-1, and NEFA.

4. To determine the effects of isoenergetic infusions of glucose and lipid on the response of leptin and other hormones (insulin, IGF-1, GH) and metabolites (glucose, NEFA, β-hydroxybutyrate) in circulation, during both early and late lactation.
5. To determine the effect of diets varying in energy and protein density during the peripubertal period on:
   (i) Blood concentrations of leptin, insulin, IGF-1, GH, glucose, NEFA, luteinizing hormone and progesterone.
   (ii) Body composition, ovarian follicular populations, and onset of puberty.

Figure 1.1. Role of leptin in the regulation of food intake and puberty in monogastric species. Inadequate body fat reserves lead to a reduction in circulating leptin concentrations which in turn leads to delayed puberty and increased food intake. During growth phase, increased deposition of body fat leads to increased circulating leptin concentrations which in turn may act to hasten the onset of puberty. Excessive concentrations of leptin in circulation may have a negative feedback effect to reduce food consumption.

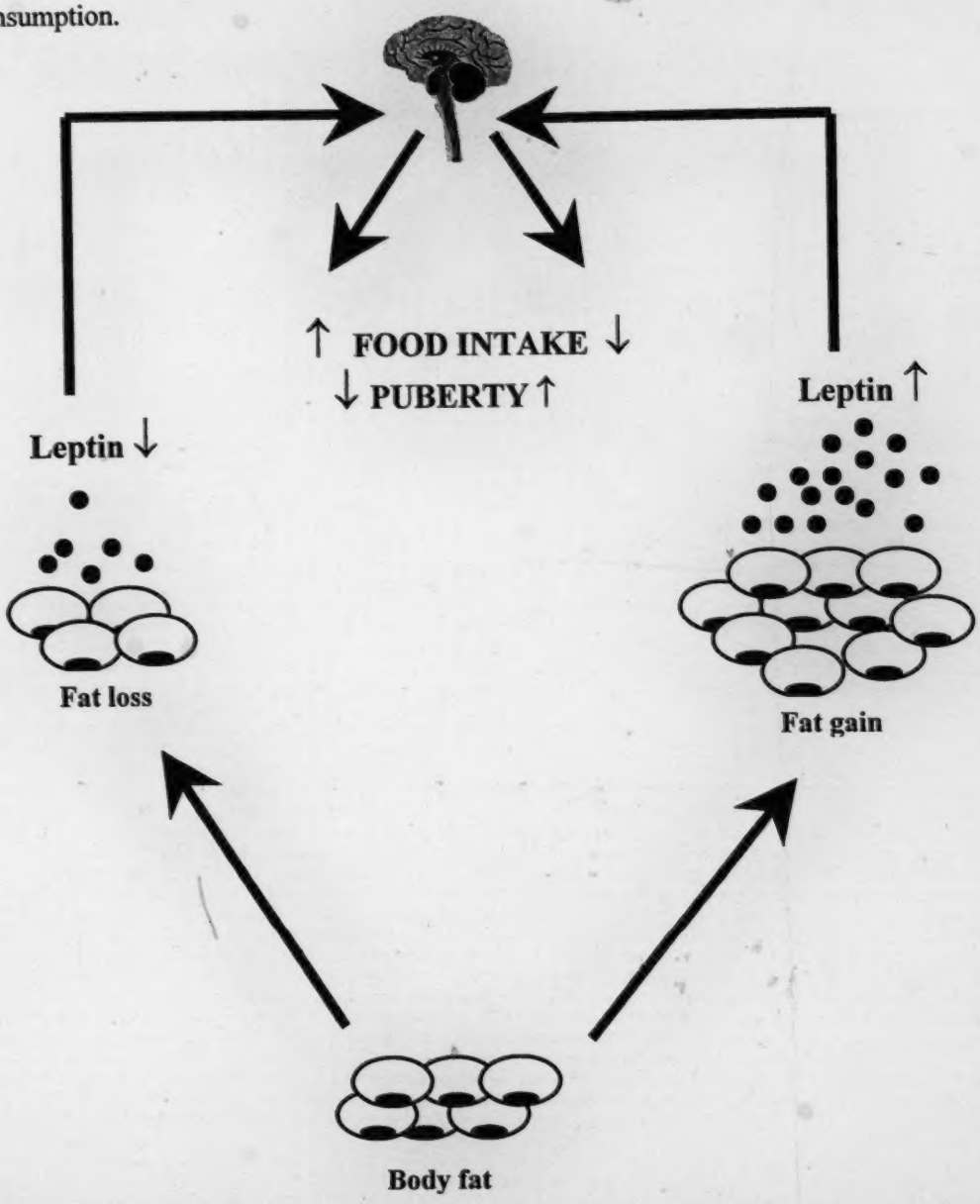

Figure 1.2. Genomic structure of the bovine leptin gene. The coding region is shown by dark boxes and the untranslated region by open boxes (from Taniguchi et al., 2002 with permission).

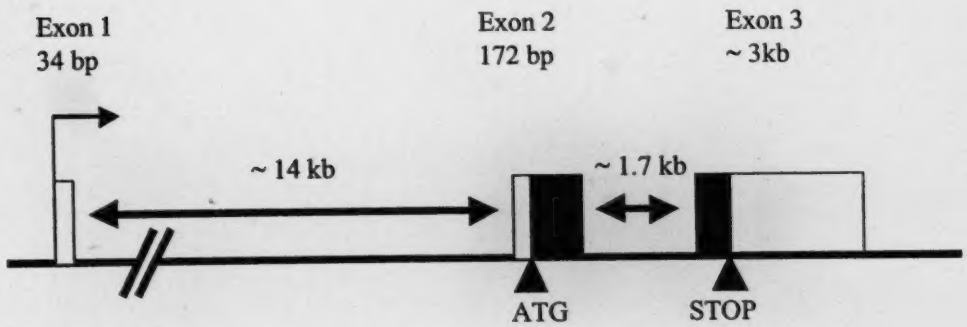

Figure 1.3. Comparison of the nucleotide sequences of leptin from domestic animals, humans, rodents and birds. The Genbank accession numbers from which the nucleotide sequences were taken are U3943, U62123, AF372504, AF102856, AF179275, AF008123, GI6678677, D49653, AF432509 for cattle, sheep, goat, pig, horse, human, mouse, rat, and chicken, respectively. The conserved regions are boxed.

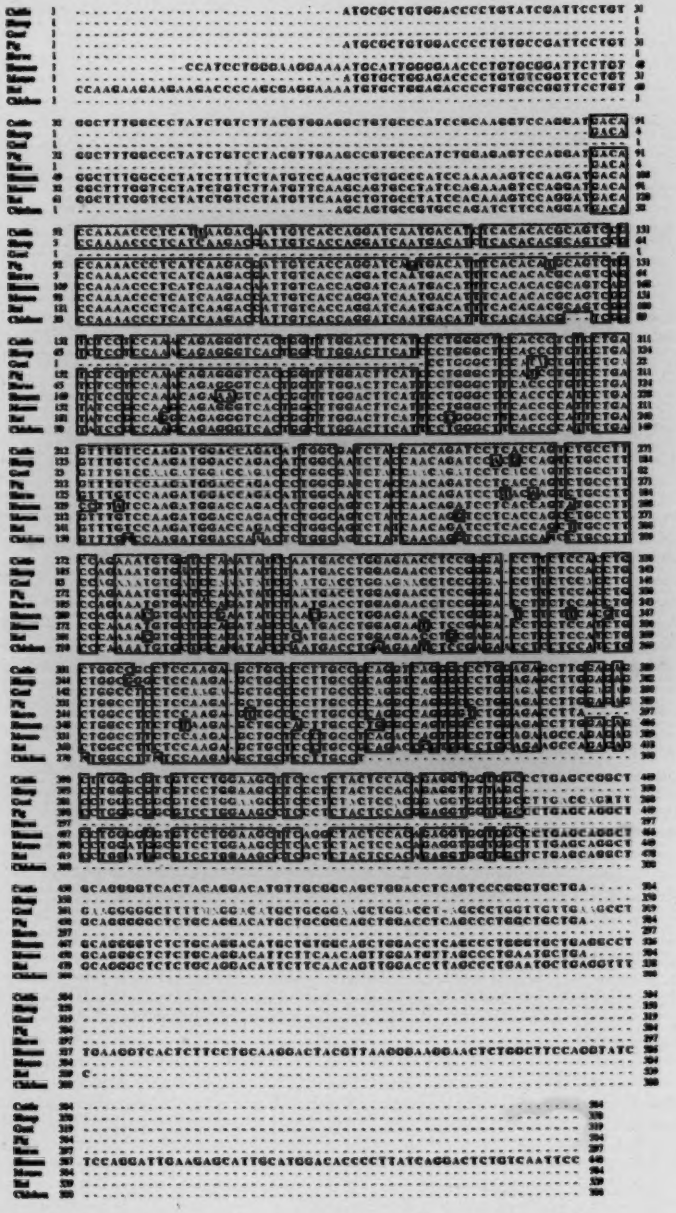

Figure 1.4. Phylogenetic tree for leptin based on aligned sequneces presented in figure 1.3. The scale bar represents 0.1 nucleotide substituition per nucleotide site.

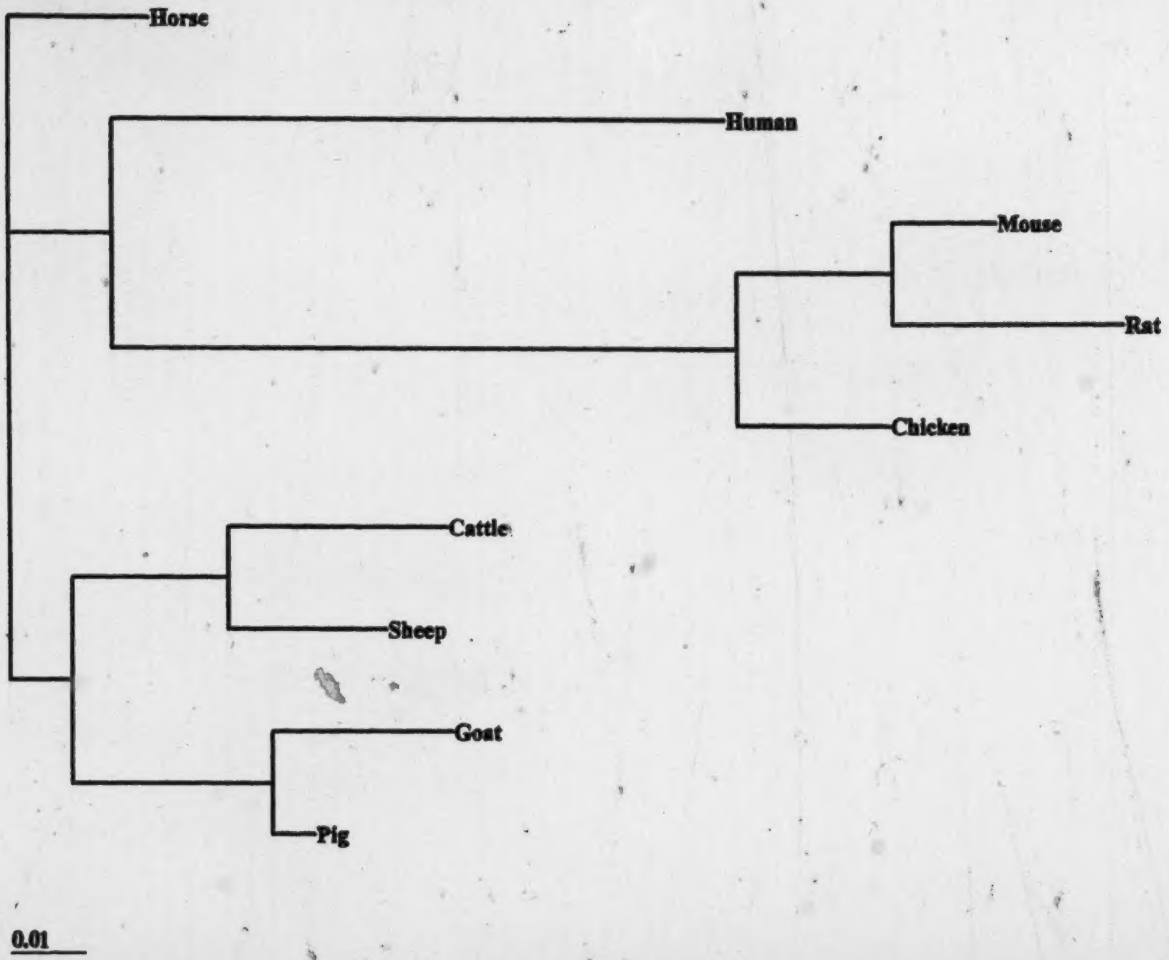

Table 1.1. Characteristics of ruminant specific assays used for quantifying leptin concentrations in circulation.

Reference	[1]Leptin	[2]Antibodies		[3]Assay	Sensitivity	[4]%CV		Recovery	Cross-	[5]Leptin (ng/ml)		[6]Fat%
		Species	Final titer		(ng/ml)	Intra	Inter	(%)	Reactivity	Cattle	Sheep	(R^2)
Blache et al. (2000a)	Bovine	Emu	1:25000	RIA	0.1	3.7	4.8	-	Sheep (+)	-	0.5-9	0.30
Kauter et al. (2000)	Bovine	Chicken	1:750	ELISA	0.5	7	15	>95	Sheep (+), Human (+)	-	3-6	-
Ehrhardt et al. (2000)	Bovine	Rabbit	1:1800	RIA	0.5	<4	<10	94–104	Sheep (+), Human (-)	4.6-6.7	6-12	0.83
Delavud et al. (2000)	Ovine	Rabbit	1:15000	RIA	0.83	13	-	102	Cattle (+), Goat (+), Human (-)	3.2-12.2	0.85-13.7	0.35
Marie et al. (2001)	Ovine	Chicken	1:2800	RIA	0.45	12	16	-	-	-	1.5-16.1	-
Thomas et al. (2001)	Ovine	Chicken	-	ELISA	1-3	13.3	18.8	95	-	-	3-21	-

[1]Recombinant leptin protein used as antigen, label, and standards; [2]Species used for raising primary antibody (Ab) to leptin and the final Ab dilution used; [3]Radioimmunoassay (RIA) or Enzyme-linked immunosorbent assay (ELISA); [4]Intra and inter assay coefficients of variation; [5]Range in cicrulating leptin concentrations as determined in each assay; [6]Variation in plasma leptin concentrations explained by body fat percentage.

Table 1.2. Summary of effects of leptin on intake, hormone, and metabolite secretion *in vivo*, and hormone secretion *in vitro* in farm animals.

Reference	Experimental model	[1]Dose and Route	[2]Effects
Liou et al. (1997)	Beef steers: perfused anterior pituitary cells	Human leptin 500 ng *(In vitro)*	(↑) LH
Barb et al. (1998)	Prepubertal gilts	Porcine leptin: 10, 50, 100 µg; ICV	(↓) Intake; (↑) GH; (-) LH, IGF-1, Gluc, Ins, NEFA, T_4
Barb (1999)	Prepubertal gilts: anterior pituitary cells	Porcine leptin: 10^{-6} to 10^{-6} *M (In vitro)*	(↑) LH & GH
Henry et al. (1999)	Ovariectomized ewe	Human leptin: 20 µg/h for 3 d; ICV	(↓) Intake & NPY in brain; (↑) Lactate & NEFA; (-) LH, FSH ,GH, Gluc, Ins
Blache et al. (2000b)	Intact (non-castrated) male sheep	Bovine leptin: 0.04 - 4 µg/h for 5 d; ICV	(↓) Intake & LH pulses; (-) FSH, GH, ACTH, Test, Prol, Gluc, Ins
Nagatani et al. (2000)	Castrated rams: Fasted/*ad libitum* fed groups	Human leptin: 50 µg/kg BW/8 h for 3 d; SC	(↑) LH & GH in fasted; (↓) Cortisol in fasted; (-)Gluc, Ins, IGF-1
Clarke et al. (2001)	Castrated rams and ewes	Human leptin: 2- 16 µg/h for 3 d; ICV	(↓) Intake- dose-dependent in spring; (-) Intake in autumn
Henry et al. (2001)	Ovariectomized ewe: Feed restriced/ *ad libitum* fed groups	Human leptin: 4 µg/h for 3 d; ICV	(↑) LH & NEFA in restricted; (↑) GH & (↓) Intake in *ad lib*; (-) Gluc, Ins, Urea, TG,

			Lactate.
Morrison et al. (2001)	Ovariectomized ewe lambs: Feed restriced/ *ad libitum* fed groups	Ovine leptin: 0-1.25 µg/h lineraly increasing doses for 8 d; ICV	(↑) GH in restricted; (↓) Intake in *ad lib*; (-) LH, Ins
Morrison et al. (2002)	Ovariectomized ewe lambs: Feed restriced/ *ad libitum* fed groups	Ovine leptin: 0-5 mg/h linearly increasing doses for 10 d; IV	(-) Intake, LH, GH, Ins, IGF-1, Cortisol, T_4
Miller et al. (2002)	Castrated rams	Ovine leptin: 0.5 to 1 mg; ICV	Autumn: (↑) LH pulse; (↓) Intake; Spring: (↑) LH pulse; (-) Intake
Amstalden et al. (2002)	Ovariectomized beef cows: Fasted/ *ad libitum* fed groups	Ovine leptin: 200 µg/h for 3 d; ICV	(↑) LH & Ins in fasted
Williams et al. (2002)	Hypothalamic/anterior pituitary explants (cows)	*In vitro*	(↑) LH
Zieba et al. (2003)	Ovariectomized beef cows: Fasted/ *ad libitum* fed groups	Ovine leptin: 0.2, 2, 20 µg/kg for 3 d; IV	(↑) LH & Ins in fasted

[1]ICV = Intracerebroventricular, IV = Intravenous, SC = Subcutaneous; [2](↑) = Increase, (↓) = Decrease, (-) = No effect, LH = Lueteinizing hormone, FSH = Follicle stimulting hormone, GH = Growth hormone, Ins = Insulin, IGF-1 = Insulin-like growth factor-1, GH = Growth hormone, ACTH = Adrenocorticotropic hormone,Test = Testosterone, Prol = Prolactin, T_3 & T_4 = Thyroid hormones, Gluc= Glucose, NEFA = Nonesterified fatty acids, TG = Triglycerides.

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CHAPTER 2

Tissue distribution of leptin and leptin receptor mRNA in the bovine[1]

2.1. Introduction

Leptin, a hormone secreted primarily from adipose tissue, has been reported to play a role in various physiological functions such as energy homeostasis, reproduction, cardiovascular, renal, immune and stress responses, and bone formation (Fruhbeck, 2001). The hormone acts through five receptor isoforms that have identical extracellular and transmembrane domains but differ in their intracellular domain. Among these isoforms, only the long-form of the receptor (Ob-Rb), with the complete intracellular domain, is fully functional and is responsible for most of the physiological effects of leptin (Tartaglia, 1997). The short-form of the receptor (Ob-Ra), with a truncated intracellular domain, has limited signal transduction capability (Bjorbaeck et al., 1997), but could be involved in leptin transport (Hileman et al., 2002) and catecholamine synthesis (Yanagihara et al., 2000). Although the expression of functional leptin receptors is highest in the central nervous system, the widespread distribution of the receptors in various peripheral tissues in monogastric species is evidence of multiple peripheral effects of leptin (Fruhbeck, 2001).

As a first step towards understanding the central and peripheral effects of leptin in cattle, it is necessary to demonstrate the gene expression of leptin and its cognate receptors in various tissues. The key objective of this study was to determine the presence of mRNA corresponding to leptin and its long-form (Ob-Rb) and short-form receptors (Ob-Ra) in various central and peripheral tissues in male Holstein calves. We also examined by semi-quantitative RT-PCR the relative expression of the leptin gene in four adipose depots.

2.2. Materials and methods

The experiment was conducted at the Metabolic Research Centre, University of

[1] A version of this chapter has been published in Journal of Dairy Science 2003; 86:2369-2372

Alberta, with all animal procedures approved by the Faculty Animal Policy and Welfare Committee. Three male Holstein calves (196.7 ± 15.62 kg BW; 5 ± 0.3 months of age) were used in this study. Within 45 min of slaughter 27 tissues were collected: subcutaneous fat, pericardial fat, perirenal fat, mesenteric fat, masseter muscle, semitendanosus muscle, endocardium, liver, adrenal cortex, spleen, kidney, testis, mesenteric lymph node, lung, aorta, rumen, abomasum, duodenum, jejunum, ileum, hypothalamus, pituitary, brain stem, cerebral cortex, cerebellar cortex, pons, and pineal gland. Samples were snap-frozen in liquid nitrogen and subsequently stored at -80^0C.

All reagents used were from Invitrogen™ life technologies (Invitrogen Canada Inc., Burlington, ON, Canada). Total RNA was isolated from pulverized tissues with TRIzol® reagent (Appendix 1) and quantified by absorbance at 260 nm in a spectrophotometer; only samples with a 260 nm : 280 nm ratio > 1.9 were used for further analysis. The reverse transcription (RT) and polymerase chain reactions (PCR) were carried out in a DNA thermocycler (PCR System 2400, Perkin Elmer, Mississauga, ON, Canada). First strand complementary DNA (cDNA) was synthesized from 2 µg of total RNA in a 20-µl reaction volume with a final concentration of 25 ng Oligo $(dT)_{12-18}$ primer, 0.5 m*M* dNTP mix, 4 µl of 5x first strand buffer, 0.01 *M* dithiothreitol, 2 U of RNaseOUT™, and 1 U of Supersript™ II RNase H reverse transcriptase. The reaction was carried out at 42^0C for 50 min, and 70^0C for 15 min. Aliquots of 2 µl of the first strand cDNA reaction were amplified in a 50-µl reaction volume containing a final concentration of 5 µl of 10x PCR buffer, 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTP mix, 2 U recombinant *Taq* DNA polymerase, and 0.4 µ*M* of each primer. Following an initial denaturation at 94^0 C for 3 min, PCR was performed for a variable number of cycles (Table 2.1) of denaturation at 94^0 C for 1 min, specific annealing temperature (Table 2.1) for 1 min, extension at 72^0 C for 2 min, and a final extension of 72^0 C for 10 min in the last cycle. The PCR products were electrophoresed, on a 2% agarose gel in Tris-borate EDTA buffer, stained with ethidium bromide, the images captured with Gel Doc 1000 system, and the PCR product density analysed with Molecular Analyst® software (Bio-Rad Laboratories, Mississauga, ON, Canada).

For each tissue and target gene, negative controls without reverse transcriptase yielded no amplification confirming that genomic DNA was not amplified (data not shown). Further, the primer pairs for leptin were designed to be located on different exons as a precaution against amplification of genomic DNA. The house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The abundance of leptin mRNA in the four adipose depots was compared by a semi-quantitative RT-PCR method. In a preliminary study, cDNA samples, in duplicate, were amplified from 12 to 34 cycles for GAPDH, and from 20 to 38 cycles for leptin. Based on these amplification plots (Figure 2.2), the optical density of leptin (r^2 = 0.97) and GAPDH (r^2 = 0.98) PCR products were each modeled as a function of number of amplification cycles by a cubic regression equation ($P<0.001$) using PRISM (GraphPad Software Inc., San Diego, California). To ensure that PCR was conducted in the linear range of amplification, cDNA samples were amplified for 28 cycles for leptin and 25 cycles for GAPDH. The PCR products were quantified as detailed above, and the ratio of leptin to GAPDH in duplicate RNA samples from the four adipose depots was analyzed by ANOVA using the MIXED procedure of SAS (1999).

2.3. Results and Discussion

The distribution of leptin and leptin receptor mRNA in various tissues is depicted in Figure 2.1. Leptin mRNA was detectable only in adipose tissue with no difference ($P > 0.10$) in transcript abundance among the four adipose depots (Figure 2.2). At the time that this manuscript was submitted to Journal of Dairy Science, the paper by Silva et al. (2002) on tissue distribution of leptin receptor mRNA in bovine tissues had not been published. In addition to confirming the expression of Ob-Ra mRNA in pituitary, liver and spleen, reported by Silva et al. (2002), we were also able to detect Ob-Ra transcripts in the adrenal cortex and brain stem. The mRNA for the long-form of the leptin receptor, Ob-Rb, was detectable in all four adipose depots, semitendanosus muscle, liver, spleen, testis, hypothalamus, pituitary, and lung, which is in agreement with the data in bovine (Silva et al., 2002; Ren et al., 2002) and swine (Lin et al., 2000). Further, Ob-Rb mRNA was also detectable in the adrenal cortex, kidney, mesenteric lymph node, aorta,

abomasum, duodenum, jejunum, ileum, brain stem, cerebral cortex, cerebellar cortex, pons, and pineal gland, but was undetectable in endocardium and massetor muscle. In accord with studies in sheep (Bonnet et al., 2002; Laud et al., 1999) and cattle (Silva et al., 2002; Smith and Sheffield, 2002) transcripts for both leptin and Ob-Rb were detectable in mammary parenchyma from a multiparous Holstein cow (data not shown).

Recent molecular evidence indicates the presence of Ob-Rb transcripts in bovine aortic cells (Parhami et al., 2001), and Ob-Ra transcripts in bovine adrenal medullary cells (Yanagihara et al., 2000). That these receptors are functional was supported by the ability of leptin to induce calcification of aortic cells through Ob-Rb, and to stimulate catecholamine synthesis in adrenal medullary cells through Ob-Ra. As the Ob-Rb and Ob-Ra primer sequences that we have used were from the above two reports we speculate that the expression of Ob-Rb and Ob-Ra transcripts in tissues that we have surveyed may have tissue-specific functional significance. The fact that most but not all tissues expressed transcripts for Ob-Rb or Ob-Ra indicates that leptin must play a role in several physiological functions. Consistent with a central role for leptin in the regulation of energy balance and reproduction (Fruhbeck, 2001), the abundance of the Ob-Rb transcript seems to be much higher in the hypothalamus and pituitary compared to other peripheral tissues. The presence of Ob-Rb mRNA in testis is suggestive of a direct role for leptin in testicular function. That leptin might be involved in lipid metabolism is suggested from the presence of Ob-Rb transcripts in adipose tissue, semitendanosus muscle, and liver. The detection of Ob-Rb message in spleen and mesenteric lymph node suggests an involvement of leptin in immune function, and presence of Ob-Rb transcript in the adrenal cortex may indicate a role for leptin in stress response and acid-base balance. Leptin may also be involved in digestion, respiration, and renal and cardiovascular functions as evidenced by the presence of Ob-Rb mRNA in various regions of the gastrointestinal tract, lung, kidney, and aorta, respectively. Although the functional significance of Ob-Ra in the liver remains to be determined, the presence of Ob-Ra mRNA in the adrenal cortex may implicate leptin in adrenal function. The Ob-Ra transcript in the brain stem may be involved in leptin transport as has been suggested for rodents (Hileman et al., 2002).

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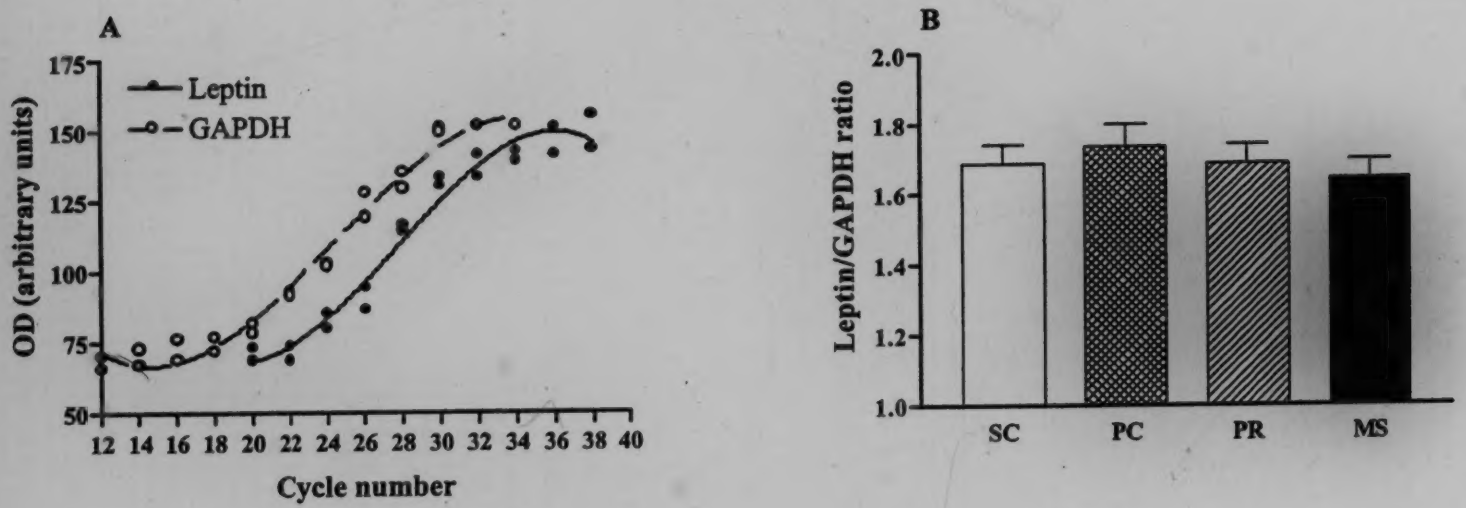
Table 2.1. Primer pairs for RT-PCR amplification of each target gene, annealing temperature (AT), number of cycles of amplification, and length (base pairs, bp) of PCR products.

Target	Primers[1]	AT (^{0}C)	Cycles	Length (bp)
Leptin	F: 5'-GTGCCCATCCGCAAGGTCC-3'	60	40	441
	R: 5'-TCAGCACCCGGGACTGAGG-3'			
Ob-Rb[2]	F: 5'-GTGCCAGCAACTACAGATGCTCTAC-3'	65	45	380
	R: 5'-AGTTCATCCAGGCCTTCTGAGAACG-3'			
Ob-Ra[3]	F: 5'-TTGAGAAGTACCAGTTCAGTC-3'	60	45	281
	R: 5'-CAAAGAATGTCCGTTCTCTTC-3'			
GAPDH[4]	F: 5'-CTGGCAAAGTGGACATTGTCGCC-3'	65	35	572
	R: 5'-CTTGGCAGCGCCGGTAGAAGC-3'			

[1]F, R: Forward and Reverse primers. Bovine–specific primer sequences for the [2]long-form of the leptin receptor (Ob-Rb) were from Parhami et al (2001) and for the [3]short-form of the leptin receptor were from Yanagihara et al. (2000). [4]Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Figure 2.1. Detection of mRNA corresponding to leptin, and the long-form (Ob-Rb) and short-form (Ob-Ra) of the leptin receptor, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in various peripheral and central tissues of a typical male Holstein calf by reverse transcription polymerase chain reaction. Lanes (Ln) 1 and 29 - 100 bp DNA ladders, Ln 2 subcutaneous fat, Ln 3 pericardial fat, Ln 4 perirenal fat, Ln 5 mesenteric fat, Ln 6 semitendanosus muscle, Ln 7 masseter muscle, Ln 8 endocardium, Ln 9 liver, Ln 10 adrenal cortex, Ln 11 spleen, Ln 12 kidney, Ln 13 testis, Ln 14 mesenteric lymph node, Ln 15 lung, Ln 16 aorta, Ln 17 rumen, Ln 18 abomasum, Ln 19 duodenum, Ln 20 jejunum, Ln 21 ileum, Ln 22 hypothalamus, Ln 23 pituitary, Ln 24 pons, Ln 25 cerebral cortex, Ln 26 cerebellar cortex, Ln 27 brain-stem, and Ln 28 pineal gland.

Figure 2.2. Semiquantitative RT-PCR for determining relative abundance of leptin mRNA in adipose depots of male Holstein calves. A cubic regression equation ($P<0.001$) was used to model the optical density (OD) of leptin ($r^2 = 0.97$) and to glyceraldehyde 3-phosphate dehydrogenase (GAPDH; $r^2 = 0.98$) PCR products as a function of number of amplification cycles (Graph A). The ratio of mRNA abundance of leptin to (GAPDH) in subcutaneous (SC), pericardial (PC), perirenal (PR), and mesenteric (MS) adipose depots (Graph B).



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CHAPTER 3

Effect of short-term fasting on plasma concentrations of leptin and other hormones and metabolites in dairy cattle[1]

3.1. Introduction

Feed intake is a complex biological phenomenon regulated by a complicated interplay of hormonal, metabolic, dietary, and environmental factors. The relative importance of these factors will vary with the physiological stage of the animal (Allen, 2000; Ingvartsen and Andersen, 2000). In order to understand the hormonal regulation of feed intake in the short-term, the experimental model that is widely used is the fasting–refeeding paradigm. Typically, hormones that initiate the hunger response are up-regulated while satiety signals are down-regulated with feed deprivation (Ahima and Flier, 2000).

Leptin, the adipose-tissue derived satiety hormone, has been reported to play an important role in regulating food intake in rodents, humans, monkeys, sheep, and pigs (Ingvartsen and Boisclair, 2001). It is proposed that the primary function of this hormone is to signal reduced food intake, with the hypothalamus sensing low leptin levels and triggering a series of adaptive neuroendocrine responses to increase food intake (Ahima and Flier, 2000). Recent studies have demonstrated that plasma leptin concentrations are reduced with fasting in prepubertal beef heifers (Amstalden et al., 2000), ovariectomized, estradiol–implanted beef cows (Amstalden et al., 2002), and and rebound on refeeding in Holstein cows (Delavaud et al., 2002). However, the fasting–induced changes in plasma leptin and its relationship to circulating concentrations of other hormones and metabolites in dairy cattle have received little attention.

We hypothesized that plasma concentrations of leptin would be reduced with short-term feed deprivation in dairy cattle with the response influenced by the physiological state of the animal. Our objectives were to determine: 1) the effects of short-term fasting and refeeding on plasma concentrations of leptin, insulin, insulin-like

[1] A version of this chapter has been accepted for Domestic Animal Endocrinology

growth factor-1 (IGF-1), nonesterified fatty acids (NEFA), glucose, and growth hormone (GH) and performance, in lactating cows, non-lactating pregnant cows, and postpubertal heifers, 2) the relationship among leptin and other hormones and metabolites under fasting-refeeding conditions and, 3) the effect of partial evacuation of rumen contents on plasma hormone and metabolite concentrations in non-lactating pregnant cows.

3.2. Materials and methods

3.2.1. Experimental Design

The experiments were conducted at the Dairy Research and Technology Centre, University of Alberta, with all animal procedures approved by the University of Alberta Animal Policy and Welfare Committee (protocol number 99 - 36 C). To minimize the potential confounding effects of diets on hormonal and metabolite response to fasting, the animals were fed, over a 2 week adaptation period, a common total mixed ration (TMR) in all three experiments with free access to water at all times. The TMR consisted of 20% barley silage, 15% alfalfa silage, 10% alfalfa hay, and 55% concentrate supplement, on dry matter basis. The nutrient composition of the, expressed as a percentage of diet dry matter was 19% crude protein, 45% neutral detergent fiber, 27% acid detergent fiber, with a metabolizable energy content of 2.67 Mcal/kg dry matter. Body weight was recorded at 24 h intervals. Body condition score (BCS; 1-5 scale) was assessed by three individuals (Edmonson et al., 1989) at the beginning and end of each study, and the mean of the three values was considered for statistical analyses.

3.2.1.1. Experiment 1. Effect of fasting on early lactation cows

Eleven multiparous (2.7 ± 0.2 lactations) Holstein cows in early lactation (55 ± 8 days in milk) were assigned at random to one of two treatment groups: *ad libitum* access to the TMR (Control, $n = 5$) or feed deprived for 48 h (Fasted, $n = 6$). The animals were housed in tie-stalls with access to water at all times. The animals were fitted with jugular catheters (polyethylene tubing I. D. 1.67 mm O. D. 2.42 mm, Intramedic PE 240, Clay Adams®, Becton Dickinson, Sparks, MD, USA) on the day before the experiment and the catheters kept patent overnight with heparinized (200 IU/ml) sterile saline. On the day of

the experiment, feed was removed (0900 h) from the fasting group and withheld for 48 h. Following fasting, the animals were allowed *ad libitum* access to feed. The control group was fed daily at 0900 h, and the amount of feed consumed was adjusted to ensure 5% orts. The cows were milked twice daily between 0400 and 0600 h and between 1600 and 1800 h. Milk yield was recorded and a sample taken at the morning milking for milk fat, protein, and lactose analyses at the Alberta Central Milk Testing Laboratory (Edmonton, AB, Canada). Energy balance was calculated according to NRC (1989) as the difference between net energy (NE) intake and NE required. NE required was calculated as NE for maintenance + NE for milk production. Net energy intake was calculated as the daily DMI multiplied by the NE_L concentration of the diet (NRC, 1989). NE for maintenance was calculated as $0.08 \times BW^{0.75}$ (kg). Animal health was monitored daily by checking body temperature, heart rate, and urinary ketones (Clini-2K Reagent strips, URI-Quick®, Stambio, USA). Blood samples were collected at 2 h intervals for the first 12 h, and at 24, 36, and 48 h following fasting and refeeding. Samples were collected on ice in 10 ml vacutainer tubes (Becton Dickinson, Sparks, MD, USA) containing EDTA or sodium heparin. Prior to, and after, collecting blood samples, the catheters were flushed with 10 to 20 ml of heparinized (20 IU/ml) sterile saline. Within 2 h of sampling, plasma was separated by centrifugation (1500 x g) and stored at -20^0 C until analyzed for hormones and metabolites.

3.2.1.2. Experiment 2. Effect of fasting, with or without partial evacuation of ruminal contents, on non-lactating pregnant cows

Four ruminally-cannulated pregnant (209-230 days in gestation) multiparous (third lactation) non-lactating Holstein cows were used in a cross-over design with two periods and two treatments: Fasting (Fasted) or Fasting with partial evacuation of rumen contents (Fasted-Evac). The animals were housed in tie-stalls with access to water at all times and fed a basal TMR (see section 2.1). During the two experimental periods, the cows were fasted for 48 h and subsequently refed. In each period, prior to fasting (0 h fast), 50% (by weight) of rumen contents were evacuated and replaced with an equal weight of normal saline in the Fasted-Evac group. The placement of jugular catheters

and blood sampling frequency were essentially the same as described in experiment 1, except that additional samples were taken at 2 h intervals from 0900 h to 2100 h on the day prior to fasting (Prefast). The amount of feed consumed was determined manually by weighing the orts at 2 h intervals for the first 12h and then at 24 h and 48 h thereafter on two consecutive days prefasting (baseline measures) and on refeeding. Each treatment period was separated by a 10 d interval to minimize carry-over effects.

3.2.1.3. Experiment 3. Effect of fasting on postpubertal heifers

In a completely randomized design, nine postpubertal Holstein heifers (16 ± 0.3 months of age) were assigned to two treatments: *ad libitum* access to a diet (Control, n = 4) or feed deprived for 72 h (Fasted, n = 5). The animals were housed in tie stalls with access to water at all times, and fed the basal TMR (see section 2.1). The weighing, jugular catheterization, and blood sampling were similar to experiment 1, except that additional blood samples and body weights were collected at 72 h of fasting and refeeding. The amount of feed consumed was determined manually by weighing the orts at 2 h intervals for the first 12h and then at 24, 48, and 72 h after feeding of fasted and control heifers.

3.2.2. Analyses

3.2.2.1. Feed analyses

Samples of TMR ingredients and orts were collected daily, dried at 55^{0}C for 72 h, ground through a 1-mm screen (Thomas-Wiley laboratory mill model 4, PA, USA) for proximate analysis. Dry matter (DM) was determined by drying samples at 110^{0}C overnight, and organic matter (OM) was calculated as weight loss upon ashing for at least 6 h at 500^{0}C.

3.2.2.2. Hormone and metabolite assays

Plasma leptin concentrations were determined by a highly sensitive ovine leptin radioimmunoassay (RIA) validated for bovine plasma (Delavaud et al., 2000). In brief, recombinant ovine leptin was used for preparation of both iodinated tracer (~20,000

cpm/tube) and standards (range 0.1 to 5 ng/tube). The primary antibody (Ab #7137) was used at a final dilution of 1:30,000. Standards were assayed in quadruplicate and samples in duplicate 200-µl volumes. Displacement of ^{125}I-labelled ovine leptin with 30 to 250 µl of bovine plasma resulted in curves that were parallel to the ovine leptin standard curve (Appendix 2). Assay sensitivity was calculated as the concentration at two standard deviations below the percentage of total binding. Sensitivity at 95 % of total binding was 0.12 ng/ml, and intra-assay coefficient of variation (CV) was 3.1 %.

Plasma concentrations of insulin were determined by a homologous double-antibody RIA as described by McGuire et al. (1995) with modifications (Appendix 3). Bovine insulin (Cat# I-5500, Sigma, St Louis, MO, USA) was used for iodination and standards (ranging from 0.009 to 4.8 ng/ml). Insulin was iodinated by the Chloramine-T method as described by Cosgrove et al. (1992). The primary antibody, guinea pig anti-bovine insulin antiserum (Cedarlane Laboratories Ltd, Mississauga, ON, Canada), was diluted in normal guinea pig serum (Cat# 2020, Calbiochem, Mississauga, ON, Canada) for a final assay tube dilution of 1:11,200. The secondary antibody, goat anti-guinea pig IgG (GAGPG, Cat# 2020, Cedarlane Laboratories Ltd, Mississauga, ON, Canada), was used at 1:10 dilution in EDTA buffer containing 3.34% polyethylene glycol. Parallelism and recoveries were evaluated in plasma pools obtained from lactating cows. Displacement of ^{125}I-labelled bovine insulin with serial dilutions of plasma resulted in curves that were parallel to bovine insulin standard curve. Recoveries of 0.07 and 0.15 ng of bovine insulin added to 1 ml plasma were 92 % and 94 % respectively. Sensitivity at 96 % of total binding was 0.009 ng/ml, and the intra-assay CV was 5.6 %.

Concentrations of IGF-1 in duplicate samples of plasma (100 µl) were determined by a heterologous double-antibody RIA following acid-ethanol extraction. The RIA procedure was essentially as described by Cosgrove et al. (1992) except that the anti-hIGF-1 primary antiserum was used at a final dilution of 1:654,000. Parallelism and recoveries were evaluated in plasma pools obtained from lactating cows. Displacement of ^{125}I-labelled human IGF-1 with 6.25, 12.5, 25, 50, and 100 µl of plasma resulted in curves that were parallel to the standard curve. For estimation of combined extraction

and assay efficiency, 30 ng of human IGF-1 was added to 50 µl of plasma prior to extraction. Radio-inert recovery was 81 %. Assay sensitivity at 79 % of total binding was 47.3 ng/ml, and the intra-assay CV was 5.1 %.

Plasma GH concentrations were determined by a homologous double-antibody RIA as described by Lalman et al. (2000). Standards were assayed in quadruplicate and samples in duplicate 200 µl volumes. Displacement of ^{125}I-labelled bovine GH with 30 to 250 µl of plasma resulted in curves that were parallel to the standard curve. Assay sensitivity at 95 % of total binding was 0.05 ng/ml., and the intra-assay CV was 2.7 %.

Plasma nonesterified fatty acids (NEFA) concentrations were determined by an enzymatic microassay kit (NEFA-C Wako Chemicals USA, Richmond, VA) validated for bovine plasma (Johnson and Peters, 1993). The intra- and inter-assay CV were 5 % and 4 % respectively. Plasma glucose concentrations were determined by enzymatic assays using kits (Sigma Chemical Co., St. Louis, MO, USA; procedure 315). The volumes of reagents and samples were modified to allow the assays to be conducted in 96-well ELISA plates and absorbance's measured with SPECTRAmax®190 Microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA). The intra- and inter-assay CV were 2.5 % and 2.6 % respectively.

3.2.2.3. Statistical analyses

Data from experiments 1 and 3 were analyzed by ANOVA for a completely randomized design using the MIXED procedure of SAS (release 8.2). Repeated measures of plasma hormones and metabolites, and other performance parameters, were analyzed using the following univariate linear mixed model:

$$Y_{ijk} = \mu + \alpha_i + \beta_{j} + \gamma_k + (\alpha\gamma)_{ik} + e_{ijk}$$

where: μ is the population mean, α_i is the fixed effect of treatment i, $\beta_{i\ j}$ is a random variable corresponding to animal j in treatment i, γ_k is the fixed effect of time k, $(\alpha\gamma)_{ik}$ is an interaction parameter corresponding to treatment i and time k, and e_{ijk} is the residual error. Based on the fit statistics of BIC criteria, the covariance structure of the repeated measurements was modeled either as compound symmetry, heterogenous compound

symmetry, first-order antedependence, or spatial power law (Littell et al., 2000). Preplanned comparisons between individual time points were made with the PDIFF option. Comparisons with $P < 0.01$ were declared highly significant, $P < 0.05$ significant, and $0.05 < P < 0.10$ were considered as trends.

Data from experiment 2 were analyzed by ANOVA for a cross-over design using the MIXED procedure of SAS. Repeated measures on plasma hormones and metabolites, and other characteristics, were analyzed using the following univariate linear mixed model:

$$Y_{ijk} = \mu + \alpha_i + \beta_{ij} + \gamma_k + (\alpha\gamma)_{ik} + \delta_l + (\alpha\delta)_{il} + e_{ijkl}$$

where: μ is the population mean, α_i is the fixed effect of treatment i, β_{ij} is a random variable corresponding to animal j in treatment i, γ_k is the fixed effect of time k, $(\alpha\gamma)_{ik}$ is an interaction parameter corresponding to treatment i and time k, δ_l is the fixed effect of period l, $(\alpha\delta)_{il}$ is an interaction parameter corresponding to treatment i and period l, and e_{ijkl} is the residual error. The covariance structure of the repeated measurements was modeled as described above for experiments 1 and 3. Preplanned comparisons between prefast and fasting values, and between fasting with and without rumen evacuation, were made with the PDIFF option. Comparisons with $P < 0.01$ were declared highly significant, $P < 0.05$ significant, and $0.05 < P < 0.10$ were considered as trends.

Pearson's correlation coefficients were used to determine the association of leptin with other plasma variables. Stepwise multiple linear regression analyses were used to determine the relative contribution of the plasma variables to the variation in plasma leptin concentrations in each experiment.

3.3. Results

Temporal changes in dry matter intake and body weight during the fasting-refeeding periods are presented in Figure 3.1. During the refeeding period, the DMI of fasted-lactating cows remained lower ($P < 0.05$) while body weight did not differ ($P > 0.10$) from controls. The DMI during the refeeding phase was similar ($P > 0.10$) to control or prefast levels upto 6 or 8 h after refeeding of heifers and dry cows,

respectively; from 10 h of refeeding onwards the DMI remained lower ($P < 0.05$) than control or prefast levels. Body weight decreased significantly ($P < 0.05$) by 48 h of fasting of cows but returned to control levels by 24 h after refeeding. In heifers, body weight decreased significantly ($P < 0.05$) by 24 h of fasting and remained low until 24 h after refeeding; thereafter there were no differences ($P > 0.10$) between control and fasted heifers. The yields of milk, protein, and lactose were decreased ($P < 0.05$) by over 55% by 48 h of fasting and remained low during the refeeding period (Table 3.1). With fasting, the percentages of milk fat gradually increased ($P < 0.05$), while lactose decreased ($P < 0.05$), but both returned to control levels by 48 h after refeeding. Milk protein percentages were similar to control values ($P > 0.10$) during the fasting period but were lower ($P < 0.05$) than control levels at 24 h after refeeding. The energy balance of fasted cows remained low ($P < 0.05$) after 24 h of fasting and did not recover to control levels during the refeeding period. The BCS values did not differ ($P > 0.10$) before and after fasting in each of the three experiments, therefore the values were pooled across treatments for each experiment. When the pooled data were compared among the physiological states, early lactation cows (2.8 ± 0.12) had lower ($P < 0.01$) BCS than non-lactating pregnant cows (3.79 ± 0.13) or postpubertal heifers (3.62 ± 0.13), but the BCS did not differ ($P > 0.10$) between non-lactating pregnant cows and postpubertal heifers.

Temporal changes in plasma hormone and metabolite concentrations under fasting-refeeding conditions in early lactation cows are shown in Figure 3.2. Relative to control values, plasma concentrations of leptin, insulin, and glucose rapidly decreased by 39%, 56%, 18%, within 2, 4, and 6 h of fasting, respectively ($P < 0.05$) and remained low thereafter for the duration of fasting. While leptin and insulin sharply returned to control levels upon refeeding ($P > 0.10$), plasma glucose lagged behind. Following this recovery phase, plasma concentrations of leptin declined again after 24 h of refeeding ($P < 0.05$). Plasma IGF-1 concentrations decreased 39% by 12 h of fasting ($P < 0.05$) but returned to control values on refeeding ($P > 0.10$). Relative to control levels, concentrations of GH in plasma increased by 36 h of fasting and remained elevated until 24 h after refeeding ($P < 0.05$). Plasma NEFA concentrations increased sharply over control levels by 4 h of

fasting ($P < 0.05$) and returned to control levels by 8 h after refeeding ($P > 0.10$).

The hormonal and metabolic changes in response to 48 h fasting of dry cows are shown in Figure 3.3. Plasma concentrations of leptin, NEFA, insulin, glucose, and IGF-1 did not differ ($P > 0.10$) between fasted-non-lactating pregnant cows (Fasted) and cows partially evacuated at the beginning of fast (Fasted-Evac). Compared to prefast values, plasma concentrations of leptin were reduced by 50% ($P < 0.05$) at 10 h of fasting in Fasted-Evac cows, and 48% at 24 h of fasting in Fasted cows, yet returned ($P > 0.10$) to prefast levels by 24 h of refeeding in Fasted cows and by 48 h in Fasted-Evac cows. Similarly, plasma insulin concentrations were reduced by 40% ($P < 0.05$) at 6 h of fasting in Fasted-Evac cows, and by 20% at 24 h of fasting in Fasted cows, and immediately returned ($P > 0.10$) to prefast levels upon refeeding. Relative to prefast values, plasma glucose concentrations were significantly reduced ($P < 0.05$) by 48 h of fasting, returned ($P > 0.10$) to prefast levels immediately on refeeding in Fasted cows, but rebounded over prefast values at 12 and 24 h of refeeding in Fasted-Evac cows. Plasma NEFA concentrations were increased ($P < 0.05$) by 12 h of fasting, and returned ($P > 0.10$) to prefast levels by 12 h of refeeding. Plasma IGF-1 concentrations were reduced 17% ($P < 0.05$) at 24 h of fasting and returned ($P > 0.10$) to prefast levels by 4 h of refeeding. Concentrations of GH in plasma were increased by 48 h of fasting and remained elevated until 4 h after refeeding ($P < 0.05$).

The effect of fasting postpubertal heifers for 72 h on hormonal and metabolic parameters is depicted in Figure 3.4. Relative to control values, plasma concentrations of leptin were reduced by 39% ($P < 0.05$) by 24 h of fasting, and remained low until 8 h of refeeding; thereafter there were no differences ($P > 0.10$) between control and fasted groups. Compared to control values, plasma concentrations of insulin were reduced by 55% ($P < 0.05$) by 10 h of fasting, and remained low until 2 h of refeeding; thereafter there were no differences ($P > 0.10$) between control and fasted animals. Plasma concentrations of glucose were reduced by 17% ($P < 0.05$) by 48 h of fasting and rapidly returned to control values on refeeding. Plasma IGF-1 concentrations were decreased by 23% ($P < 0.05$) at 24 h of fasting and remained low until 48 after refeeding. Although plasma GH concentrations were numerically greater with fasting, there were no

significant differences between fasted and control heifers ($P > 0.10$). Plasma NEFA concentrations rapidly increased above control values by 4 h of fasting ($P < 0.05$) and remained elevated until 24 h after refeeding; thereafter, there were no differences ($P > 0.10$) between NEFA concentrations in control vs fasted heifers.

The correlations among leptin, glucose, insulin, NEFA, and IGF-1 are shown in Table 3.2. In all three experiments, plasma concentrations of leptin were positively correlated ($P < 0.001$) with IGF-1, and negatively with NEFA and GH. Further, leptin was positively correlated ($P < 0.05$) with glucose in early-lactation cows, and with insulin in non-lactating cows and postpubertal heifers. Stepwise multiple regression analysis revealed that of the plasma variables considered: IGF-1 (18%) and glucose (8%) together explained 26% of the variation in plasma leptin concentrations in early lactation cows; IGF-1 (14%) and insulin (2%) contributed to 16% of the variation in plasma leptin concentrations in dry cows; and IGF-1 (14%) and insulin (4%) together explained 18% of the variation in plasma leptin concentrations in heifers.

3.4. Discussion

In ruminants, the reduction in feed intake following a period of fasting could be due to alterations in ruminal fermentation (Fluharty et al., 1996), electrolyte balance (Cole, 2000), hormonal and (or) metabolite adaptations. One or more of these factors might have been involved in inducing post-fast satiety in our experiments. In non-lactating pregnant cows, a 50% dilution of ruminal contents at the beginning of fast had no effect on post-fast DMI. Similarly, exchanging 50% of ruminal contents between fed and fasted sheep did not affect post-fast DMI (Cole, 1991). Postfast DMI was not influenced by 50% dilution of rumen contents, therefore, it is unlikely that ruminal contents or rumen fill *per se* have a major impact on post-fast DMI in dairy cows.

Plasma hormones and metabolites exhibited marked fluctuations in our fasting-refeeding experiments and these alterations were particularly rapid in early lactation cows. We provide evidence here that plasma leptin concentrations are acutely responsive to short-term fasting and refeeding in dairy cattle at various physiological states. This is in general agreement with recent studies in prepubertal beef heifers (Amstalden et al.,

2000), beef cows (Amstalden et al., 2002), and in sheep (Daniel et al., 2002; Marie et al., 2001). In addition, we also report that the leptin response to fasting is dependent on the body condition and physiological state of the animal, and that partial dilution of ruminal contents has no affect on plasma leptin concentrations in cattle. As expected, fasting resulted in reduction in plasma concentrations of insulin, glucose, and IGF-1, and elevation in NEFA, similar to other reports in dairy (McGuire et al., 1995; Frohli and Blum, 1988; McCann and Hansel, 1986) and beef cattle (Amstalden et al., 2002; Spicer et al., 1992; Ward et al., 1992).

Compared to the sharp reduction (~40%) of plasma leptin concentrations within 2 h of fasting in lactating cows, the response in non-lactating pregnant cows and heifers was more blunted as leptin concentrations did not decrease significantly until approximately 12 h in non-lactating pregnant cows and 24 h in heifers. These changes occurred in the absence of any reduction in body weight or BCS, and despite the fact that lactating animals had lower BCS and lower basal leptin concentrations compared to non-lactating pregnant cows or heifers. Similar to leptin, the temporal changes in plasma concentrations of glucose, NEFA, insulin, and IGF-1 were relatively blunted in non-lactating pregnant cows and heifers compared to the rapid changes observed in early lactation animals. Although not directly comparable to our observations, underfeeding (at 60% of maintenance energy requirements) for three weeks was found to reduce plasma leptin concentrations in fat but not in thin dry non-pregnant adult Holstein cows (Delavau et al., 2002). The duration and intensity of feed restriction, as well as differences in physiological status, might explain the discrepancies between these studies. Potential limitations in comparing hormonal and metabolic responses across our three experiments, however, are the different experimental designs employed and the confounding effects of stage of lactation and body condition. Regardless, our observations are consistent with recent studies on hormonal and metabolic adaptations to fasting in lean and obese human subjects. In obese humans the fasting-induced reduction in plasma concentrations of insulin, leptin, and glucose, and elevation in NEFA, were reported to be blunted compared to lean subjects (Heptulla et al., 2001; Horowitz et al., 2001; Landt et al., 2001; Klein et al., 2000). It is also likely that the differential leptin response to fasting in our

study reflects the differences in nutrient requirements; the responses were less rapid in animals with lower requirements (postpubertal heifers < pregnant non-lactating cows < early lactation cows).

As milk production explains nearly 45% of the variation in DMI in high producing cows (Roseler, 1997), it might be expected that cows in early lactation would exhibit rapid adaptations to short-term insults to energy balance such as feed deprivation by reducing energy losses (such as milk production) and by down-regulating the production of satiety signals. In accord with this logic, our early lactation cows adapted rapidly by reducing the production of milk and milk components. The sharp reduction in either plasma leptin, insulin, or glucose, or the rapid increase in plasma NEFA, during the early starvation period, could serve as afferent signals to the hypothalamus about the severity of negative energy balance, as reported in rats (Dallman et al., 1999). Extrapolating from a recent study in sheep (Adam et al., 2002), the fasting-induced reduction in plasma leptin concentrations could be expected to increase hunger through an up-regulation of orexigenic signals such as neuropeptide Y and agouti-related transcript and a down-regulation of anorexigenic neuropeptides such as cocaine- and amphetamine-regulated transcript. The issue that needs to be addressed, however, is whether the differential leptin response to fasting, as we have demonstrated, would be reflected in differences in expression of the above mentioned neuropeptides in animals of varying physiological stages.

We provide here evidence of differential leptin response to refeeding of fasted animals. Plasma leptin concentrations rapidly returned to control levels on refeeding of early-lactation cows unlike the slower response observed in non-lactating pregnant cows and heifers. . However, the decline in leptin following 24 h of refeeding was unexpected, and might be due to the lower DMI of refed animals, increased clearance of the hormone from circulation, or due to an inhibitory effect of other signals on leptin secretion. In all three of our experiments, plasma insulin, leptin, and IGF-1 concentrations were similar to control levels during the immediate refeeding period. This argues against a role for these hormonal signals in inducing satiety in cattle during the early refeeding phase. In support of this, a postprandial reduction in plasma leptin concentrations was also reported in well-

fed cows (Delavaud et al., 2002). However, in contrast to these short-term homeostatic adaptations, it is likely these that these hormones might play an important role in the long-term regulation of feed intake (Ingvartsen and Andersen, 2000).

The mechanisms through which fasting decreases circulating concentrations of leptin are not clear. In monogastrics, the fasting-induced reduction in leptin concentrations is believed to be due to increased sympathetic activity, increased ketones and free fatty acids, and/or due to a reduction in insulin and glucose concentrations (Fried et al., 2000; Coleman and Herrmann, 1999). Further, in infusion of β-agonists was reported to reduce plasma leptin concentrations in cattle (Chilliard et al., 2001). In our studies, the positive correlation of leptin with glucose, insulin, and IGF-1, suggest that these plasma variables might act as leptin secretagogues in cattle. Further, of the plasma variables considered, IGF-1 and glucose were the strongest predictors of plasma leptin concentrations in early lactation cows, while IGF-1 and insulin contributed significantly to the variation in plasma leptin concentrations in non-lactating pregnant cows and heifers. In fact, in all three experiments, plasma insulin concentrations either coincided or preceded the changes in leptin concentrations during fasting and refeeding. These changes in insulin occurred before significant alterations in glucose, IGF-1, or NEFA, indicating that insulin might act as an early cue for leptin regulation in cattle. In support of our observations, insulin dose-dependently increased leptin mRNA expression *in vitro* from bovine subcutaneous adipose tissue explants, while leptin mRNA abundance was strongly correlated with IGF-1 mRNA *in vivo* (Houseknecht et al., 2000). The removal of anti-lipolytic effects of insulin and IGF-1, and increased sensitivity to elevated GH, under fasting conditions could be expected to result in an enhanced lipolytic response and an elevation of plasma NEFA concentrations as observed in our studies. The fasting-induced increase in plasma NEFA, and the negative correlation between NEFA and leptin, provide evidence that the fasting-induced lipolysis, and subsequent intracellular accumulation of free fatty acids, might act to down-regulate leptin gene expression, as demonstrated in rats (Arai et al., 2002; Renstch and Chiesi, 1996). The increase in plasma GH concentrations with fasting of lactating and non-lactating pregnant cows is consistent with other reports on dairy cattle (McGuire et al., 1995; Athanasiou and

Phillips, 1978; Trenkle, 1976). It is conceivable that this increase in plasma GH, in concert with NEFA, mediated the reduction in leptin concentrations with fasting. In support of an inhibitory role of GH on leptin production, GH was shown to attenuate the stimulatory effects of insulin and dexamethasone on leptin expression in bovine adipose tissue explants (Houseknecht et al., 2000), and transgenic sheep with enhanced GH production were reported to have low plasma leptin concentrations (Kadokawa et al., 2003a,b).

In conclusion, we report that plasma leptin concentrations are acutely responsive to fasting and refeeding in dairy cattle. The response, however, appeared to differ among cattle of varying physiological status. It is unlikely that leptin is involved in mediating post-fast satiety in cattle; on the contrary, the fasting-induced acute hypo-leptinemia in early lactating cows could be an adaptive response to increase feed intake during the refeeding period. The positive association of leptin with changes in plasma glucose, insulin, and IGF-1 suggest that they might mediate the effects of fasting on leptin production. Future studies on the direct effects of these hormonal and metabolic mediators on leptin expression and release, under acute fasting-refeeding conditions, should prove useful in identifying the nutritional cues that trigger leptin production in cattle in the short-term.

Table 3.1. Effect of short-term fasting and refeeding on milk yield and composition, and energy balance in dairy cows. Treatment differences ($P < 0.05$) between control (Con) and fasted-refed (Fast) animals at each time point are indicated by different superscripts (a, b).

		Fasting			Refeeding		SEM
	Time (h)	0	24	48	72	96	
Milk yield	Con	18.26	16.3	19.44[a]	18.77[a]	21.54[a]	1.84
(kg)	Fast	21.83	17.78	8.65[b]	8.18[b]	12.65[b]	1.68
Fat %	Con	4.39	4.39	4.16[a]	3.29[a]	3.98	0.49
	Fast	4.47	5.46	7.53[b]	4.99[b]	4.83	0.45
Fat yield	Con	0.78	0.72	0.77	0.59	0.85	0.10
(kg)	Fast	0.96	0.97	0.65	0.41	0.62	0.09
Protein %	Con	2.96	2.96	3.04	3.09[a]	3.07	0.08
	Fast	2.98	2.83	3.13	2.37[b]	2.71	0.07
Protein yield	Con	0.54	0.48	0.59[a]	0.58[a]	0.66	0.06
(kg)	Fast	0.65	0.51	0.27[b]	0.19[b]	0.34	0.05
Lactose %	Con	4.64	4.64[a]	4.58[a]	4.69[a]	4.65	0.69
	Fast	4.67	4.16[b]	3.77[b]	4.44[b]	4.67	0.06
Lactose yield	Con	0.85	0.75	0.89[a]	0.87[a]	0.99[a]	0.08
(kg)	Fast	1.02	0.74	0.33[b]	0.36[b]	0.59[b]	0.08
Energy Balance	Con	20.22	14.61[a]	13.99[a]	17.17[a]	16.43[a]	2.69
(Mcal/day)	Fast	16.63	-23.81[b]	-17.43[b]	3.16[b]	4.12[b]	2.46

Table 3.2. Pearson correlation coefficients of plasma leptin with glucose, nonesterified fatty acids (NEFA), insulin, insulin-like growth factor-1 (IGF-1) and growth hormone (GH) in each of the fasting-refeeding experiments.

	Early Lactation cows	Non-lactating pregnant cows	Postpubertal heifers
Glucose	0.34***	0.22**	0.03
NEFA	-0.37***	-0.44***	-0.46***
Insulin	0.29	0.27***	0.19*
IGF-1	0.43***	0.27***	0.37***
GH	-0.24***	-0.30***	-0.39***

*$P < 0.05$

**$P < 0.01$

***$P < 0.001$

Figure 3.1. Effect of short term fasting and refeeding on feed intake and body weight in lactating dairy cows (A, B), non-lactating pregnant dairy cows (C, D), and postpubertal dairy heifers (E, F). In non-lactating pregnant cows, rumen contents were either partially evacuated (Fasted-Evac) or not evacuated (Fasted) at the beginning of fasting. Figures A, C, and E depict the amount of dry matter consumed (DMI) on refeeding of fasted animals. Figures B, D, and F depict changes in body weight during both fasting and refeeding periods. Treatment differences ($P < 0.05$) between control and fasted-refed animals are indicated by asterisks (*), and between prefast and fast values by letters (a, b). Values indicate means (± SEM).

Figure 3.2. Mean plasma concentrations (± SEM) of leptin (A), nonesterified fatty acids (NEFA; B), insulin (C), glucose (D), insulin-like growth factor-1 (IGF-1; E), and growth hormone (GH; F) in early lactation cows fasted for 48 h and subsequently refed. Treatment differences ($P < 0.05$) between control and fasted-refed cows are indicated by asterisks (*).

Figure 3.3. Mean plasma concentrations (± SEM) of leptin (A), nonesterified fatty acids (NEFA; B), insulin (C), glucose (D), insulin-like growth factor-1 (IGF-1; E), and growth hormone (GH; F) in non-lactating pregnant cows fasted for 48 h and subsequently refed. The rumen contents were either partially evacuated (Fasted-Evac) or not evacuated (Fasted) at the beginning of fasting. Treatment differences ($P < 0.05$) between prefast and fast values are indicated by letters (a, b).

Figure 3.4. Mean plasma concentrations (± SEM) of leptin (A), nonesterified fatty acids (NEFA; B), insulin (C), glucose (D), insulin-like growth factor-1 (IGF-1; E), and growth hormone (GH; F) in postpubertal heifers fasted for 72 h and subsequently refed. Treatment differences ($P < 0.05$) between control and fasted-refed heifers are indicated by asterisks (*).

Figure 3.1

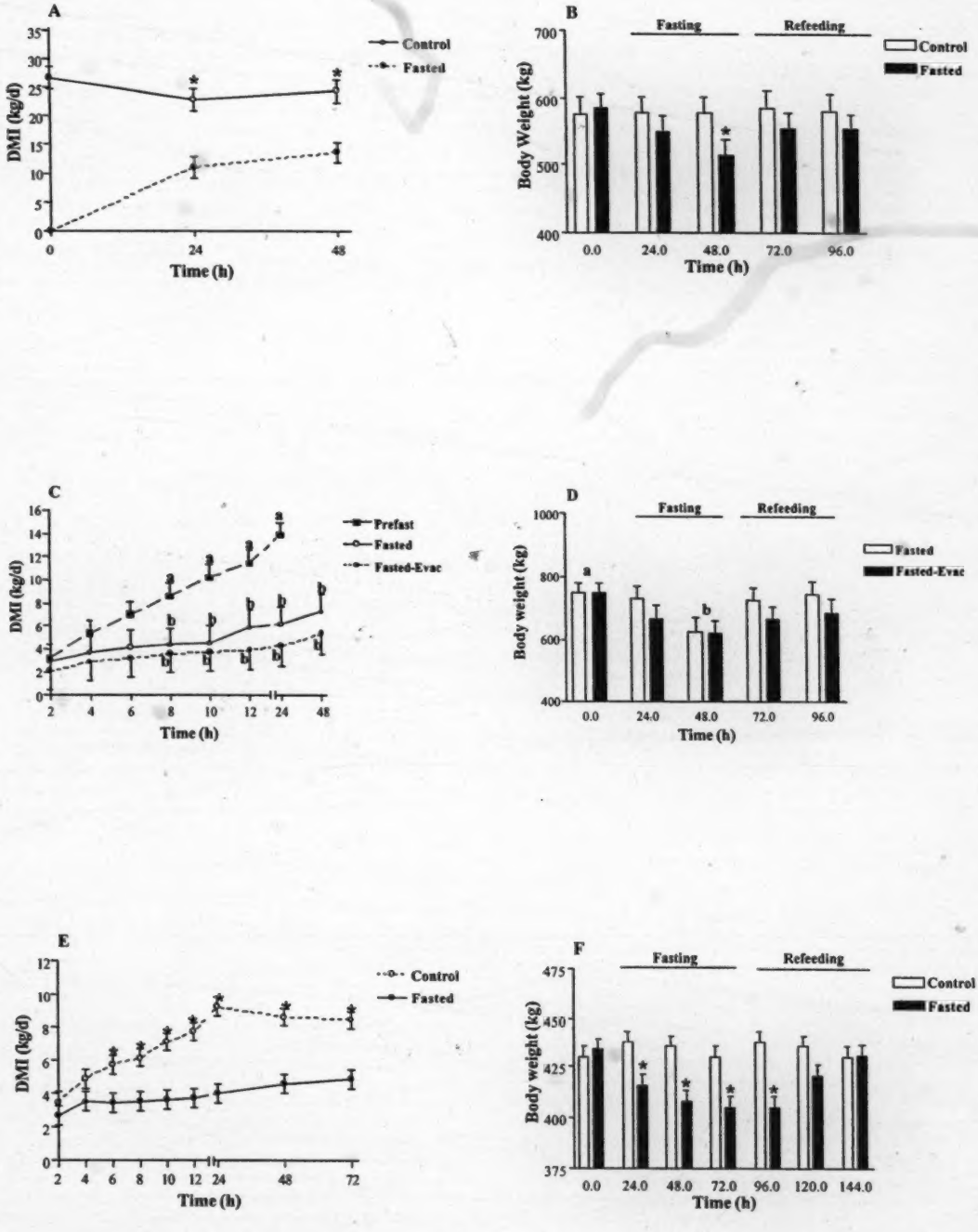
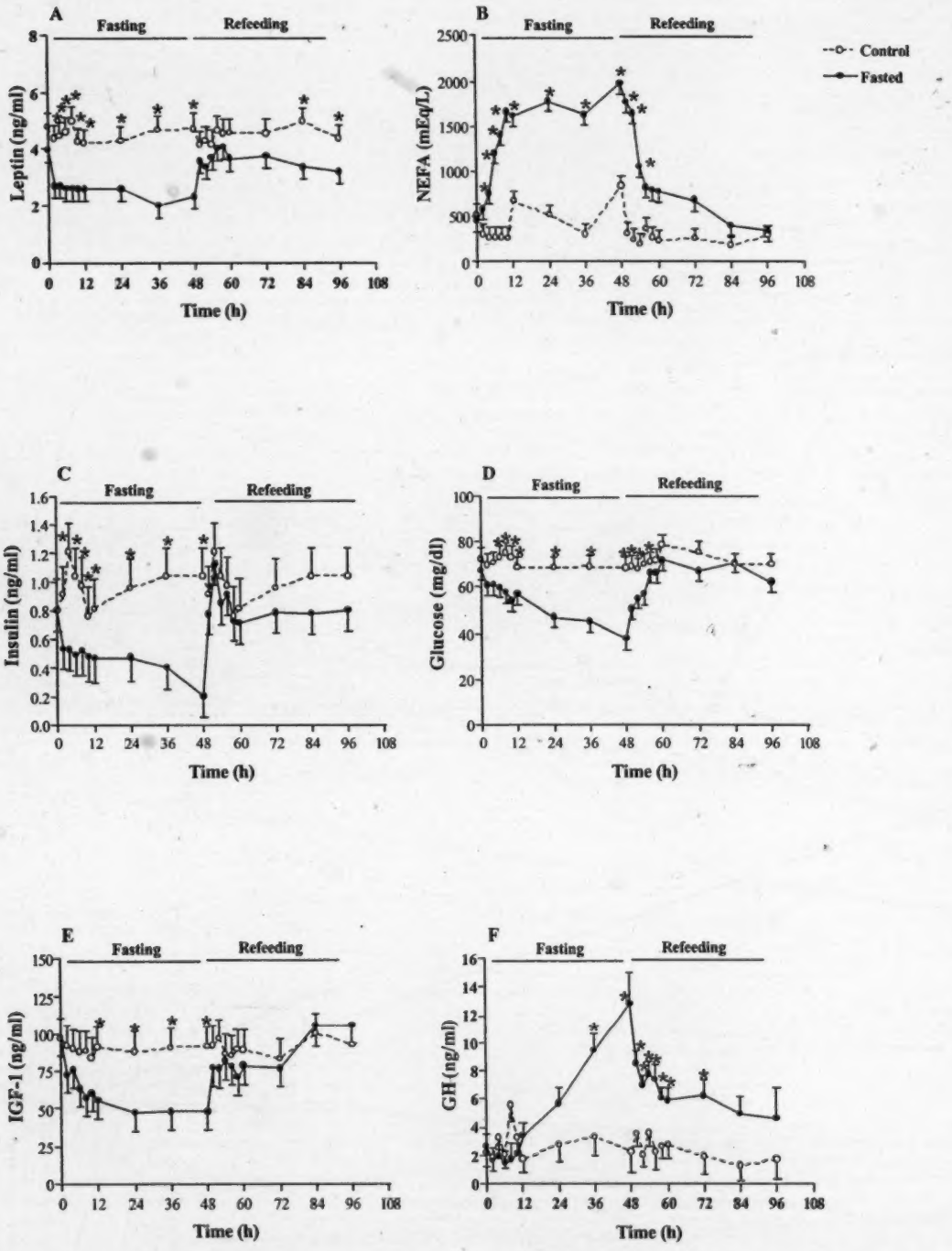

A
DMI (kg/d)
Time (h)
Control
Fasted
B
Body Weight (kg)
Fasting
Refeeding
Control
Fasted
Time (h)
C
DMI (kg/d)
Prefast
Fasted
Fasted-Evac
Time (h)
D
Body weight (kg)
Fasting
Refeeding
Fasted
Fasted-Evac
Time (h)
E
DMI (kg/d)
Control
Fasted
Time (h)
F
Body weight (kg)
Fasting
Refeeding
Control
Fasted
Time (h)

Figure 3.2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Fasting
Refeeding
Leptin (ng/ml)
Time (h)
B
Fasting
Refeeding
NEFA (mEq/L)
Time (h)
Control
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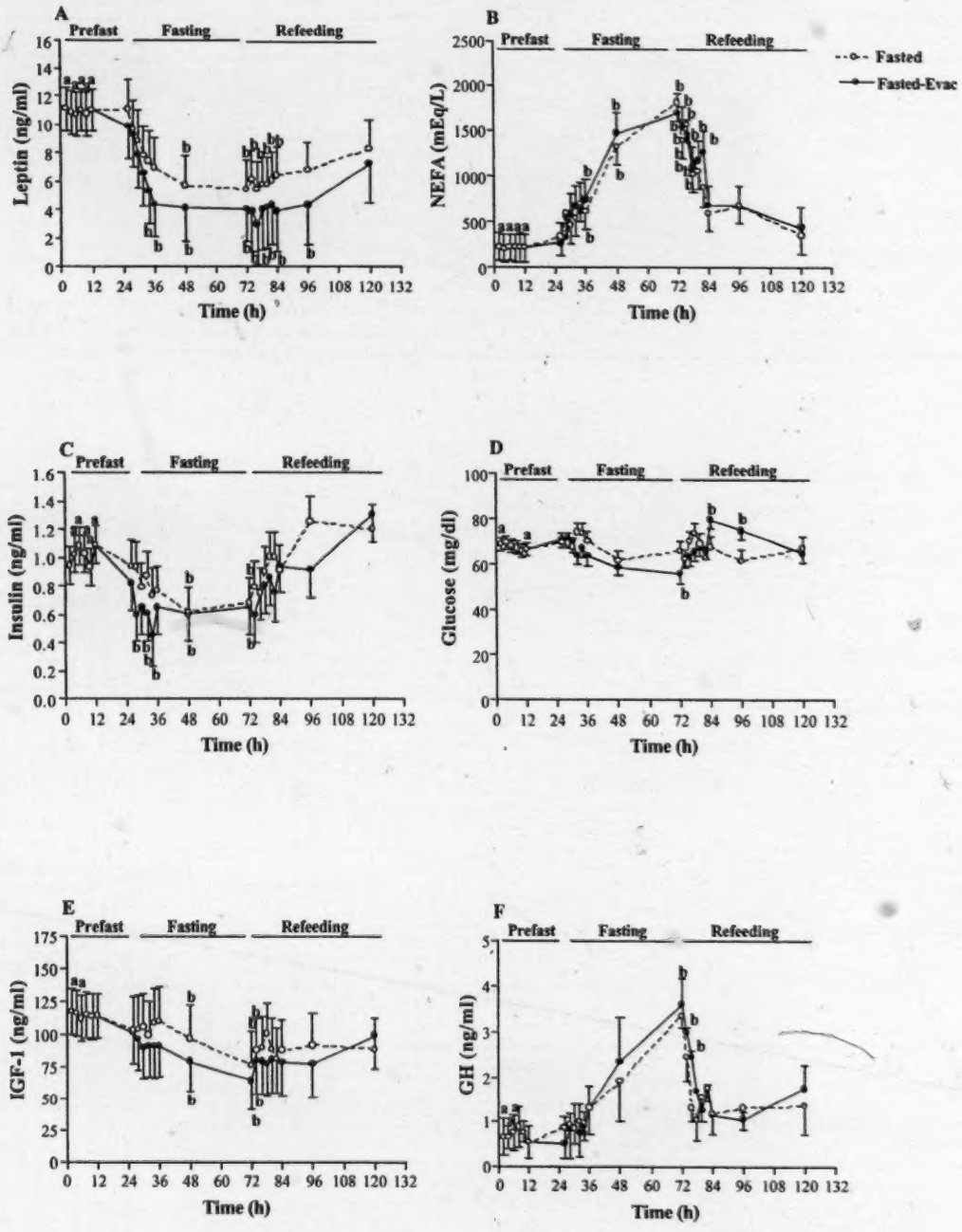
C
Fasting
Refeeding
Insulin (ng/ml)
Time (h)
D
Fasting
Refeeding
Glucose (mg/dl)
Time (h)
E
Fasting
Refeeding
IGF-1 (ng/ml)
Time (h)
F
Fasting
Refeeding
GH (ng/ml)
Time (h)

Figure 3.3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Prefast
Fasting
Refeeding
Leptin (ng/ml)
Time (h)
B
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Fasting
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NEFA (mEq/L)
Time (h)
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Fasted-Evac
C
Prefast
Fasting
Refeeding
Insulin (ng/ml)
Time (h)
D
Prefast
Fasting
Refeeding
Glucose (mg/dl)
Time (h)
E
Prefast
Fasting
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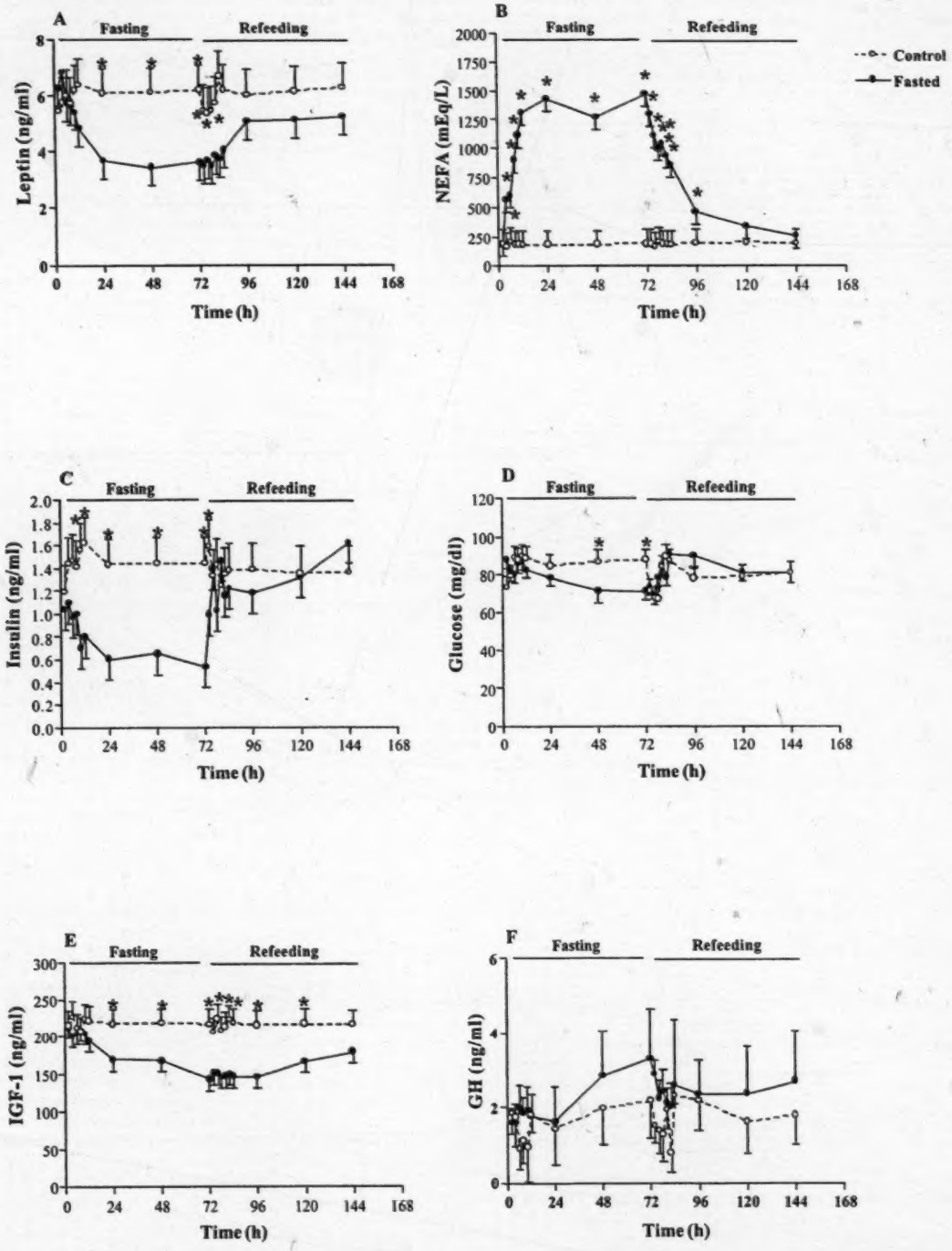
Time (h)
F
Prefast
Fasting
Refeeding
GH (ng/ml)
Time (h)

Figure 3.4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Fasting
Refeeding
Leptin (ng/ml)
Time (h)
B
Fasting
Refeeding
NEFA (mEq/L)
Time (h)
Control
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Fasting
Refeeding
Insulin (ng/ml)
Time (h)
D
Fasting
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Glucose (mg/dl)
Time (h)
E
Fasting
Refeeding
IGF-1 (ng/ml)
Time (h)
F
Fasting
Refeeding
GH (ng/ml)
Time (h)

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CHAPTER 4

Effect of feeding or abomasal infusion of canola oil on nutrient digestion, milk composition, mRNA abundance and plasma concentrations of leptin and cholecystokinin in dairy cows[1]

4.1. Introduction

Feeding high levels of fat has been reported to depress feed intake in dairy cattle. Some of the factors that are involved in this response include impaired rumen fermentation, inhibition of fiber digestibility leading to increased rumen fill, palatability, profile of fatty acids (FA) reaching the intestine, release of hormonal signals from the gut and oxidation of fat in the liver (Allen, 2000). Among these factors, the profile of long-chain fatty acids reaching the intestine seems to be critical because, in a majority of studies in which rumen-protected lipid supplements were fed, dry matter intake is consistently reduced (Allen, 2000). However, the hormonal and (or) metabolic signals mediating the hypophagic effects of fat are not clearly understood.

The fat-induced increase in circulating concentrations of gut hormones such as cholecystokinin (CCK), pancreatic polypeptide (Choi and Palmquist, 1996; Choi et al., 2000; Suominen et al., 1998), glucagons, and (or) glucagon-like peptide-1 are proposed to mediate satiety in cattle (Suominen et al., 1998; Benson and Reynolds, 2001). Leptin, a satiety signal from adipose tissue, could also be involved in mediating the hypophagic effects of fat. In rodents, feeding high fat diets was shown to increase the concentration of leptin in blood (Coleman and Herrmann, 1999). Further, the response is dependent on fatty acid chain length with dietary polyunsaturated long-chain FA stimulating leptin secretion independent of adipose tissue mass (Cha and Jones, 1998). Thus, a plethora of hormonal and metabolic signals could be operating to depress DMI when fat is supplemented to dairy cattle. The relative importance of these signals, the time-scale over which they operate, and the response of these signals to the method of fat

[1] Two manuscripts from this chapter have been accepted for Journal of Dairy Research

supplementation and to the degree of saturation of FA supplied to the intestine, are still unknown.

We hypothesized that the depression in dry matter intake that occurs with fat supplementation is dependent on the fatty acid composition of the digesta reaching the intestine, and that these satiety effects may be mediated through cholecystokinin and (or) leptin. To compare the relative importance of ruminal versus postruminal effects of fat on intake, and other parameters, fat was either included in the diet or infused postruminally. Postruminal infusion allows more precise control over the flow of specific fatty acids to the small intestine than would be possible with the use of "protected" lipid supplements as none of these supplements completely prevent hydrolysis and biohydrogenation of dietary fatty acids. Further, biohydrogenation of unsaturated fatty acids in the rumen would alter the profile of fatty acids (FA's) reaching the intestine and their subsequent transfer into milk. Thus, the FA composition of milk is influenced by the composition and digestibility of the FA reaching the intestine.

Our objectives were to determine the effect of feeding *versus* postruminal supply of large amounts of canola oil on: 1) nutrient digestibility, duodenal flows of fatty acids, milk yield and fatty acid composition, 2) mRNA abundance of cholecystokinin (CCK) gene in the duodenum, and of mRNA abundance of leptin, acetyl-coA carboxylase (ACC), and lipoprotein lipase (LPL) genes in adipose tissue and 3) blood concentrations of CCK, leptin, insulin, IGF-1, glucose and fatty acids,

4.2. Materials and methods

4.2.1. Cows, experimental design and treatments

Five ruminally and duodenally cannulated multiparous Holstein cows were used in an incomplete latin square design with three treatments and three 16 day (d) periods. The cows were in late lactation and averaged 249 days in milk (SE 14 d) at the beginning of the experiment. The animals were housed in tie stalls with access to feed and water at all times. The cows were fed once daily at 0900 hours (h) to ensure 5% orts; they were exercised and milked twice daily between 0400 and 0600, and between 1600 and 1800 h.

All procedures were approved by the University of Alberta Animal Policy and Welfare Committee.

The three treatments consisted of 1) Control (CON): basal total mixed ration (TMR), 2) Control + abomasal infusion (INF) of 1 kg/d of canola oil (Champion Feed Services Ltd, Edmonton, Canada), and 3) Control + canola oil mixed with feed at 1kg/d (FED). The basal TMR was formulated to meet or exceed the NRC (1989) requirements for late lactation cows (Table 4.1). For the FED treatment, we anticipated that there would be losses of canola oil during mixing with other feed ingredients in the data rangers, at the time of delivery of feed to each animal, and also in the orts. As a precaution against such losses, and to minimize potential effects of the oil on palatability, an excess 10% (~1.1 kg) of oil per animal was added to the concentrate prior to mixing with forage in the data rangers. All cows had infusion lines placed in the abomasum, but the control and oil-fed cows did not receive any infusate. The oil was continuously pumped for 18 h (at ~60g/h) by means of a Masterflex® peristaltic pump (Labcor Inc., QC, Canada) with a standard drive and four EasyloadII® pump heads. The Tygon®tubing (L/S®16) which conveyed the infusate was anchored in the abomasum with a plastisol flange attached to a perforated polypropylene bottle. The placement and patency of the infusion lines in the abomasum was confirmed every two days. In each period the animals were adapted to the oil, which was either supplemented in the feed or continuously infused into the abomasum, by gradually increasing the amount by 250 g/d over a 4-d period.

4.2.2. Sampling

Feed consumption during the last 7 d of each period was used to measure dry matter intake (DMI). Samples of TMR, ingredients, and orts were collected during the last 4 d of each period and composited by cow within period. Body weight and body condition score (BCS) were recorded once at the end of each period. Body weights were recorded between 0700 and 0800, which was after milking and before feeding.

Beginning on d 15, ruminal fluid samples were collected at 0850, 0930, 1000, 1030, 1100, 1200, 1300, 1500, 1700, 1900, 2100, 2300, 0100, 0300, 0500 and 0700 h. The samples were filtered through a strainer and pH of each sample was determined immediately using a glass electrode. An aliquot (~4 ml) of sample was acidified with 1 ml of 25% H_3PO_4 and frozen for determination of volatile fatty acids (VFA) and ammonia nitrogen.

For determining nutrient flows and digestibilities, 10 g of Cr_2O_3 marker was administered into the rumen twice daily, at 0600 and 1800, during the last 10 d of each period (Elliott et al., 1997). Fecal grab samples were collected at 12-h intervals from d 12 to d 16 of each period and, composited on an equal wet weight basis by animal within period. Samples of duodenal digesta (~250 ml) were collected at 4 h intervals over a 48 h period on d 15 to d 16, the pH was determined, and samples were composited for each animal within a period on an equal wet weight basis. Daily milk yield was recorded, and milk samples were collected on d 14 to d 16. Individual samples were analyzed for milk fat, protein and lactose by infrared analysis at the Alberta Central Milk Testing Laboratory (Edmonton, AB, Canada). On each sampling day, the samples collected in the morning and evening were composited as a percentage of yield and stored at -20^0C for later fatty acid analysis.

The cows were fitted with jugular catheters (polyethylene tubing ID 1.67 mm, OD 2.42 mm, Intramedic PE 240, Clay Adams®, Becton Dickinson, Sparks, MD, USA) on d 13 of each period for blood sampling and patency was maintained overnight with 200 IU/ml of heparinized saline. Blood samples were collected on d 14 at 0850, 0930, 1000, 1100, 1300, 1500, 1700, 1900, and 2100 into EDTA vacutainer tubes (Becton Dickinson), plasma was separated by centrifugation at 1500 x g for 20 min and stored at -20^0C.

Duodenal biopsies were taken with a fiberoptic endoscope (Olympus CF, type 1B, Olympus America Inc., Melville, NY, USA) equipped with a biopsy forceps by a procedure validated in our laboratory (Suominen et al., 1998). Biopsies were taken on d 14 of each period at 30 min before start of feeding or infusion (0 h sample), and subsequently at 4, 6, and 8 h after the beginning of feeding or infusion. At each time

point a total of four biopsies were taken at approximately 40 cm distal to the abomasal sphincter. On d 16 of each period, adipose tissue biopsies were taken from the tail-head region under local anesthesia. Tissue samples were immediately rinsed in sterile PBS, snap frozen in liquid nitrogen, and stored at -80^{0}C.

4.2.3. Chemical analyses

Samples of feed, orts, and digesta were dried at 60^{0}C for 72 h, and ground through a 1-mm screen (Thomas-Wiley laboratory mill model 4, PA, USA). Dry matter was determined by drying samples at 110^{0}C overnight, and OM was calculated as weight loss upon ashing for at least 6 h at 500^{0}C. Samples were analyzed for CP (6.25 x N; Leco FP-428 nitrogen determinator, Leco® Corporation, MI, USA), NDF, ADF, lignin (Ankom filter bag technique®, Ankom Company, NY, USA) and gross energy (Leco® Automatic Calorimeter, AC300, Leco Corporation, MI, USA). Ruminal fluid concentrations of VFA and NH_3N were determined according to Khorasani et al. (1996). Fecal and duodenal samples were analyzed for Cr according to the procedure of Fenton and Fenton (1979).

Milk fat extraction and transmethylation were performed according to the procedure of Chouinard et al. (1999). Milk fatty acid methyl esters (FAME) were analyzed on a Varian 3600 gas chromatograph equipped with a septum programmable injector and flame ionization detector. The FAME were separated on a BPX70 fused silica capillary column (50m x 0.32 mm, SGE column, Australia). The injector temperature was increased from 70^{0}C to 230^{0}C at 150^{0}C/min and held for 17 min. The detector temperature was held constant at 230^{0}C. The column conditions were: initial column temperature held at 50^{0}C for 0.1 min, increased at 25^{0}C/min to 170^{0}C and held for 1 min, increased from 170^{0}C to 180^{0}C at 2^{0}C/min, then raised to 230^{0}C at 10^{0}C/min and held for 3 min. The flow rate of the gases were: helium carrier gas at 1ml/min at 21 psi, helium make-up gas at 30 ml/min, hydrogen at 30 ml/min, and air at 300 ml/min. Identification of peaks was based on comparison with retention times of standard FAME (GLC #85, GLC #411, Methyl *trans*-11 18:1, and *cis*-9 *trans*-11 CLA, Nuchek Prep Inc.,

MN, USA). Peak areas of each fatty acid were expressed as a proportion of the total FAME detected.

Feed, duodenal, and fecal FAME (Appendix 5) were prepared by a modification of the procedures of Sukhija and Palmquist (1988) and Chin et al. (1992). To 0.5 g of sample in screw capped 16 x 125 mm test tubes, 1 ml of hexane, 1ml of heptadecanoic acid internal standard ($C_{17:0}$: 4 mg/ml in hexane), and 3 ml of fresh 4% methanolic HCl were added. The tubes were tightly capped, vortexed, and heated for 1 h at 60^0C. Methylation with 4% methanolic HCL at 60^0C has been reported to convert only 5% of the *cis*-9 *trans*-11 CLA isomer to *trans*-9 *trans*-11/ *trans*–10 *trans-12* CLA isomers (Chin et al., 1992). After cooling, 5 ml of 6% K_2CO_3 and 2 ml of hexane were added, the tubes were vortexed and centrifuged. To the hexane extract 1 g of Na_2SO_4 and 1 g of activated charcoal were added, the tubes vortexed and centrifuged, and the clear hexane layer was transferred into GC vials. GC analysis was carried out as described for milk FAME.
Nutrient intake was calculated by multiplying DM intake by nutrient concentration. Fatty acid intake for cows abomasally infused with canola oil (INF) was calculated by adding the intake of fatty acids from basal diet to the amount of fatty acids infused. Duodenal and total tract flows of dry matter (DM) were calculated by dividing the amount of Cr dosed per day (10 g) by the Cr concentration in duodenal and fecal samples, respectively. DM flows were multiplied by nutrient concentration in duodenal or fecal samples to obtain duodenal or total flows of nutrients. Digestibilities were calculated as: (nutrient intake - nutrient flow)/nutrient intake. Energy balance was calculated according to NRC (1989) as the difference between net energy (NE) intake and NE required. NE required was calculated as NE for maintenance + NE for milk production. Net energy intake was calculated as the daily DMI multiplied by the NE_L concentration of the diet. Diet NE_L was determined from table values, and the assigned table values for fat were 9.3 kcal/g. NE for maintenance was calculated as 0.08 x $BW^{0.75}$ (kg).

4.2.4. Gene expression analyses

The mRNA abundance of CCK in duodenal samples, and leptin, acetyl-coA carboxylase (ACC), and lipoprotein lipase (LPL) in adipose tissue samples were analyzed

by a semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) method similar to the procedure described in Chapter 2. All reagents used were from Invitrogen™ life technologies (Invitrogen Canada Inc., Burlington, ON, Canada). Total RNA was isolated from pulverized adipose tissues with TRIzol® reagent and quantified by absorbance at 260 nm in a spectophotometer; only samples with a 260 nm : 280 nm ratio > 1.9 were used for further analysis. All four duodenal biopsies collected at each time point were composited for each animal prior to isolation of RNA (Appendix 4) as described by Suominen et al. (1998). The RT-PCR were carried out in a DNA thermocycler (PCR System 2400, Perkin Elmer, Mississauga, ON, Canada). First strand complementary DNA (cDNA) was synthesized from 2 μg of total RNA in a 20-μl reaction volume with a final concentration of 25 ng oligo $(dT)_{12\text{-}18}$ primer, 0.5 m*M* dNTP mix, 4 μl of 5x first strand buffer, 0.01 *M* dithiothreitol, 2 U of RNaseOUT™, and 1 U of Supersript™ II RNase H reverse transcriptase. The reaction was carried out at 42^0C for 50 min, and 70^0C for 15 min. Aliquots of 2 μl of the first strand cDNA reaction were amplified in a 50-μl reaction volume containing a final concentration of 5 μl of 10x PCR buffer, 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTP mix, 2 U recombinant *Taq* DNA polymerase, and 0.4 μ*M* of each primer. Following an initial denaturation at 94^0C for 3 min, PCR was performed for a variable number of cycles (Table 4.9) of denaturation at 94^0C for 1 min, specific annealing temperature (Table 4.9) for 1 min, extension at 72^0C for 2 min, and a final extension at 72^0C for 10 min in the last cycle. The PCR products were electrophoresed, on a 2% agarose gel in Tris-borate EDTA buffer, stained with ethidium bromide, the images captured with Gel Doc 1000 system and the PCR product density analyzed with Molecular Analyst® software (Bio-Rad Laboratories, Mississauga, ON, Canada). To confirm identity of the PCR products, the products were purified with QIAquick PCR Purification Kit® (Cat # 28104, QIAGEN Inc, Mississauga, ON, Canada) and sequenced by the DNA Core Laboratory, Department of Biochemistry, University of Alberta.

For each sample and target gene, negative controls without reverse transcriptase yielded no amplification confirming that genomic DNA was not amplified (data not

shown). Further, each primer of the primer pair for leptin was designed to be located on a different exon as a precaution against amplification of genomic DNA. The house-keeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. In a preliminary study, cDNA samples, in duplicate, were amplified from 10 to 40 cycles for each target gene. The optical density of each PCR product was determined and then modeled as a function of number of amplification cycles by fitting polynomial regression curves using PRISM (GraphPad Software Inc., San Diego, CA, USA). From these curves, the cycle number from approximately the middle part of the linear range was selected for each target gene (Table 4.9). For each cycle number selected, linearity between the amount of cDNA input and the optical density of resulting PCR product was determined (data not shown). The ratio of the optical density of the PCR product of each target gene to that of GAPDH was calculated before statistical analyses.

4.2.5. Hormone and metabolite assays

Plasma concentrations of CCK were determined with a double-antibody radioimmunoassay (RIA) kit (EURIA-CCK, ALPCO Diagnostics, American Laboratory Products Company, Windham, NH, USA) with modifications. For extracting CCK from plasma, 2 ml of 98% ethanol was added to 1 ml of plasma sample in 12 x 75 mm glass tubes. The mixture was vortexed and centrifuged at 2,000 x g at 4^0C for 15 min. The supernatant was transferred into another tube, dried overnight in a rotary evaporator and the extracts stored at -20^0C until assayed. The dried extracts were reconstituted with 1 ml of assay buffer and duplicate 200-µl extracts were assayed according to the manufacturer's recommendations. The standard curve was modified to include 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, and 25 pmol/l of CCK-8 standard. Parallelism and recovery were evaluated in plasma pools obtained from lactating cows. Displacement of ^{125}I-CCK-8 sulphate with extracts of serially diluted plasma resulted in curves that were parallel to the standard curve. Addition of 200 µl of 50 pmol/l CCK standard to 800 µl of

plasma resulted in 78.6% recovery. Assay sensitivity, intra- and inter-assay coefficients of variation were 0.12 pmol/l, 4.4% and 8.4%, respectively.

Plasma concentrations of leptin, insulin, and insulin-like growth factor-1 (IGF-1) were determined by radioimmunoassays according to the procedures described in detail in Chapter 3. Intra-assay coefficient of variation for leptin, insulin, IGF-1were 2.9%, 5.1%, and 6.6% respectively. Plasma glucose concentrations were determined by an enzymatic assay as decribed in Chapter 3. The intra- and inter-assay coefficients of variation of glucose were 2.2% and 1.6%, respectively. Fatty acid methyl esters (FAME) from plasma and adipose tissue samples were prepared by direct methylation using methanolic HCL as described above for feed and digesta samples.

4.2.6. Statistical analyses

Data for nutrient intake and digestion, milk composition, and rumen VFA, were analyzed by ANOVA for a 3 X 5 incomplete Latin square design using the general linear models procedure of SAS (1999). Intake and production variables were reduced to means for each cow in each period. The model for analyzing these variables and digestibility was (Hicks, 1999):

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + e_{ijk}$$

where μ is the population mean, α_i is a population parameter corresponding to treatment i (i = 1, 2, 3), β_j is a random variable corresponding to animal j (j = 1, 2, 3, 4, 5), γ_k is a parameter corresponding to period k (k = 1, 2, 3) and e_{ijk} is the residual error. The main effects of cow, period and treatment were tested using the residual error. The hourly data on ruminal parameters (pH, VFA and ammonia) were analyzed as a split-plot in time using the following model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\gamma)_{ik} + (\alpha\beta\gamma)_{ijk} + \delta_l + (\alpha\delta)_{il} + (\beta\delta)_{jl} + (\gamma\delta)_{jk} + e_{ijkl}$$

where $(\alpha\gamma)_{ik}$ is the effect of treatment by period interaction, $(\alpha\beta\gamma)_{ijk}$ is the treatment by cow by period interaction, δ_l is the effect of time (l = 1 to 16), $(\alpha\delta)_{il}$ is the effect of treatment by time interaction, $(\beta\delta)_{jl}$ is the effect of cow by time interaction, $(\gamma\delta)_{jk}$ is the effect of period by time interaction, and e_{ijkl} is the residual error. The main effects of

cow, period and diet were tested using the whole-plot error term $(\alpha\beta\gamma)_{ijk}$, and the treatment by time interaction $(\alpha\delta)_{il}$ was tested using the residual error term (e_{ijkl}). Mean separation was performed using the LSMEANS statement with the PDIFF option after Tukey-Kramer's adjustment. Stepwise regression analysis was used for determining the relative contribution of individual FA flows at duodenum to variation in DMI.

Repeated measures on plasma hormones and metabolites, and gene expression, were analyzed using the following univariate linear mixed model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + e_{ijk}$$

where μ is the population mean, α_i is a population parameter corresponding to treatment i, β_j is the fixed effect of period j, γ_k is a parameter corresponding to time k, $(\alpha\beta)_{ij}$ is the effect of treatment by period interaction, $(\alpha\gamma)_{ik}$ is the effect of treatment by time interaction, and e_{ijkl} is the residual error. The Kenward-Roger procedure was used for approximating the degrees of freedom. Animal nested in treatment and period was considered as the subject on which repeated measures were taken and covariance structures modeled. Based on the smallest values of fit statistics for AICC and BIC criteria, the covariance structure of the unequally spaced repeated measurements for each variable was modeled either as compound symmetry, heterogenous compound symmetry, first-order antedependence, or spatial power law (Littell et al., 2000). Preplanned treatment comparisons were made with the PDIFF option and declared significant at $P < 0.05$.

4.3. Results

4.3.1. Intake, duodenal flows, and total tract digestibility of nutrients

The ingredient and chemical composition of the basal diet and the FA composition of canola oil are presented in Tables 4.1 and 4.2, respectively. Nutrient intake, duodenal flows, and total tract digestibility are presented in Table 4.3. Relative to CON treatment, abomasal infusion of oil (INF) caused a significant ($P < 0.05$) reduction in DMI. Further, intake when expressed as a percentage of body weight was also reduced ($P < 0.05$) with INF compared to CON or FED (CON 2.57%, FED 2.58%, INF 2.07%,

SE 0.10). Body weight (CON 544, FED 563, INF 548, SE 9.0, kg) and body condition score (CON 2.48, FED 2.66, INF 2.29, SE 0.19) did not differ ($P > 0.10$) among treatments. Energy balance (Mcal/d) did not differ ($P > 0.10$) among treatments (CON 7.38, FED 11.64, INF 7.77, SE 1.46). With INF, intakes of OM, NDF and ADF were lower ($P < 0.05$) compared to CON or FED, and intakes of CP were lower ($P < 0.05$) than FED. Intakes of DM, OM, CP, NDF, and ADF did not differ ($P > 0.10$) between CON and FED treatments. Duodenal flows of DM and OM were reduced with INF compared to CON or FED ($P = 0.05$). Except for greater ruminal digestion and lower duodenal flows of CP for FED than CON ($P < 0.05$), there were no differences among treatments ($P > 0.10$) for either ruminal or total tract digestion of DM, OM, NDF, ADF and cellulose.

4.3.2. Ruminal fermentation

Treatment x time interaction was not significant ($P > 0.10$) for ruminal parameters, therefore, means across sampling times are presented in Table 4.4. Overall mean concentrations of ammonia and pH were not different ($P > 0.10$) among treatments. The total molar concentrations of VFA were reduced ($P < 0.05$) with INF compared to CON or FED. The molar proportion of propionate decreased ($P < 0.05$) for cows receiving the INF compared to CON. The molar proportions of other VFA, and acetate : propionate ratio, did not differ ($P > 0.10$) among treatments.

4.3.3. Intake, duodenal flows, and total tract digestibility of fatty acids

Intake and duodenal flows of fatty acids are presented in Table 4.5. Compared with CON, both INF and FED resulted in higher ($P < 0.001$) total FA intake. Intakes of total FA and individual FA did not differ ($P > 0.10$) between FED and INF. Compared to CON, both FED and INF resulted in nearly a 2-fold increase ($P < 0.01$) in total FA flow at duodenum. The increase in duodenal flow of unsaturated FA ($P < 0.05$) with FED compared to CON was primarily due to a 4-fold increase of both *cis* and *trans* isomers of 18:1; and the increased flow of saturated FA was due to greater flows of 18:0 (+ 150%) and 16:0 (+ 77%). Duodenal flow of *trans*-11 18:1 was greater (+ 367%) with FED

compared to INF ($P < 0.05$), and flows of *cis*-9 *trans*-11 18:2 (CLA) were only measurable with FED but undetectable with CON or INF. As expected, greater flows of 18:1*n*-9, 18:2*n*-6, and 18:3*n*-3 with INF ($P < 0.01$) contributed to the increase in unsaturated FA flow and in unsaturated : saturated FA ratio when compared to CON or FED ($P < 0.05$). Although the duodenal flows of unsaturated and saturated FA were greater with FED compared to CON ($P < 0.05$), the unsaturated : saturated FA ratio did not differ ($P > 0.10$).

Total tract digestibility and postruminal absorption of FA are presented in Table 4.6. Total tract digestibility of FA did not differ ($P > 0.10$) between CON and FED except for reduced ($P < 0.05$) digestibility of 18:0 (- 16%) and 15:0 (- 22%) with FED. However, absorption (g/day) of all FA was greater ($P < 0.05$) with FED relative to CON. Compared to CON, with INF, digestibilities of 15:0, 16:0, 18:0, and 18:1*n*-9 were reduced ($P < 0.05$) by nearly 20% leading to a reduction in total FA digestibility by 10%. The postruminal absorption (g/d) of 18:1*n*-9, 18:2*n*-6, and 18:3*n*-3 were greater ($P < 0.05$) with INF than CON or FED. Compared with CON, FED resulted in increased ($P < 0.05$) postruminal absorption of 16:0, 18:0 and 18:1*n*-9. The total amount of FA absorbed postruminally was nearly 2-fold greater ($P < 0.001$) with FED or INF relative to CON. In comparison with FED, digestibilities of 16:0, 18:1*n*-9 and total FA, and postruminal absorption of 18:0 were reduced ($P < 0.05$) with INF.

4.3.4. Milk yield and fatty acid composition

The yields of milk and milk components did not differ ($P > 0.10$) between CON and FED, but milk fat percentage was reduced ($P < 0.05$) with FED (Table 4.7). Relative to CON, INF resulted in a 39% drop in milk fat percentage ($P = 0.007$) and a 35% reduction ($P = 0.01$) in milk fat yield.

Composition of milk FAME are presented in Table 4.8. Compared to CON, FED or INF resulted in significant ($P < 0.001$) reductions in saturated and medium chain FA (11:0 to 17:0), and increases in monounsaturated long chain FA. Relative to CON, saturated FA were reduced by 16% with FED, and 30% with INF. There were no treatment differences ($P > 0.10$) in the proportions of individual short chain (4:0 to 10:0)

FA (data not shown). The proportions of medium chain FA were reduced by 26% with FED, and 36% with INF compared to CON. This decrease was primarily due to a reduction of 14:0 by 18% with FED ($P < 0.01$), 32% with INF ($P < 0.001$); and a reduction ($P < 0.001$) of 16:0 by 29% with FED and 39% with INF. Relative to CON, 18:0 was increased 29% with FED ($P < 0.01$) but was reduced by 17% with INF ($P < 0.05$). The proportions of 18:2*n*-6 and 18:3*n*-3 were similar between CON and FED ($P > 0.10$), but were increased by 267% and 270% respectively with INF ($P < 0.001$). Total 18:1 increased ($P < 0.001$) by more than 40% with FED and INF relative to CON. Compared to CON, among the 18:1 isomers, 18:1*n*-9 was increased ($P < 0.001$) by 26% with FED and 43% with INF, whereas *trans*-11 18:1 increased 194% with FED ($P < 0.001$) but was not affected by INF ($P > 0.10$). The proportion of the CLA isomer *cis*-9 *trans*-11 18:2 was increased by 166% with FED compared to CON or INF ($P < 0.001$).

4.3.5. Gene expression

The mRNA abundance of CCK in duodenal biopsies was greater ($P < 0.05$) with FED and INF compared to CON at the four time-points considered (Least square means and SEM are presented in Figure 4.2). The abundance of transcripts for leptin, ACC and LPL in subcutaneous adipose tissue were similar ($P > 0.10$) among treatments (Least square means and SEM are presented in Figure 4.2).

4.3.6. Plasma hormones

Plasma concentrations of leptin, insulin and IGF-I did not differ ($P > 0.10$) among treatments (Figure 4.1). There was a significant treatment x time interaction ($P < 0.01$) for plasma CCK concentrations. In FED and INF treated cows (vs CON), plasma CCK concentrations were greater ($P < 0.05$) at 1, 4, 6, 8, 10, and 12 h after the beginning of feeding or infusion of canola oil. At 12 h, plasma CCK concentrations were greater in INF vs FED cows ($P < 0.05$).

4.3.7. Plasma glucose and fatty acids, and adipose fatty acid composition

Treatment x time interaction was not significant ($P > 0.10$) for plasma glucose and fatty acid concentrations; therefore, overall means are presented in Table 4.10. Plasma concentrations of glucose did not differ ($P > 0.10$) among treatments. Relative to CON, total fatty acid concentrations in plasma were increased ($P < 0.05$) with both FED (+ 81%) and INF (+ 89%). The proportions of medium chain FA were, in general, reduced by fat supplementation. Compared to CON, the proportions of 10:0, 12:0 and 14:0 were reduced ($P < 0.05$) by 30%, 81% and 33% with FED, and by 77%, 80% and 78% with INF, respectively. With INF, the proportion of 16: 0 was reduced ($P < 0.05$) by 48% compared to CON or FED, whereas the proportions of 18:1*n*-9, 18:1*n*-11 and 18:2*n*-6, were increased by 90%, 52% and 6% relative to CON, and by 54%, 34%, and 26% relative to FED, respectively. With FED, compared to CON or INF, the proportion of 18:0 was increased ($P < 0.05$) by 32% whereas *trans*-11 18:1 and *cis*-9 *trans*-11 18:2 (CLA) were increased nearly 4-fold.

The fatty acid composition of subcutaneous adipose tissue is shown in Table 4.11. With INF, the proportions of 16:0 and 16:1 were reduced ($P < 0.05$) by 12% and 17% compared to CON and by 6% and 8% relative to FED, respectively; whereas 18:1*n*-9 was increased ($P < 0.05$) by 11% and 7% compared to CON and FED, respectively. Relative to CON, with FED the proportions of 16:0 and 16:1 were reduced ($P < 0.05$) by 6% and 10%, respectively.

4.4. Discussion

4.4.1.Intake, digestion and milk composition

We compared the effects of a single lipid source, canola oil, on nutrient intake, digestion, and milk composition of cows by either supplementing the oil in the feed (FED) or infusing the same amount of oil into the abomasum (INF). In this study, rumen fermentation was relatively stable with FED as evidenced by the lack of effect on rumen pH, ammonia nitrogen, VFA's and fiber digestibility. Although duodenal flows of total unsaturated and saturated FA were greater with FED than CON, their ratios were similar. Therefore, the relative stability of the rumen and postruminal environment with FED might explain the lack of any adverse effects on DMI and concurs with results from a

recent study with canola oil supplementation (DePeters et al., 2001) but not others (Jenkins, 1999; Jenkins et al., 2000).

The reduction in feed intake with postruminal oil infusion in our study is in agreement with a majority of reports in which fat was infused postruminally or protected from rumen metabolism. The amount of oil infused in our study, and the associated 2.6 kg reduction in feed intake, is comparable to data from other laboratories. For example, duodenal infusion of canola oil at 1.1 kg/d (Gagliostro and Chilliard, 1991) or 0.7 kg/d (Ottou et al., 1995) resulted in a 2.6 kg or 1.8 kg drop in DMI respectively, while no changes in DMI was reported with ruminal or abomasal infusion of 0.33 kg/d of canola oil (DePeters et al., 2001). Protection of canola from rumen digestion has also resulted in DMI reduction in some studies (Jenkins, 1999; Jenkins et al., 2000) but not in others (Enjalbert et al., 1997; Khorasani and Kennelly, 1998). As expected, flows of unsaturated and total FA, and the ratio of unsaturated to saturated FA, at the duodenum were much greater with INF than CON or FED. Stepwise regression analysis revealed that of the total FA flows at the duodenum, 18:1*n*-9 and 18:2*n*-6 together explained 89% of the variation in DMI, with 18:2*n*-6 contributing 81% and 18:1*n*-9 to 8% of the total variation in DMI ($P < 0.01$). According to Bremmer et al. (1998) the amount of 18:1*n*-9, 18:2*n*-6 and 18:3*n*-3 infused into the abomasum together explained 64% of the variation in DMI. Therefore, in addition to the total amount of unsaturated FA reaching the intestine, the ratio of unsaturated: saturated FA, and increased flows of 18:1*n*-9 and 18:2*n*-6, might have contributed to the hypophagic effects of abomasal canola oil infusion in our study. Potential hormonal and/or metabolic mediators involved in these effects hypophagic effects of fat are discussed in the next section.

The reduction in molar concentrations of propionate and total VFA with INF compared to CON or FED treatments could possibly be due to lower substrate availability for fermentation. These changes in VFA patterns with postruminal oil infusion were similar to another report (Gagliostro and Chilliard, 1991), but not others (Bremmer et al., 1998). It is likely that these changes in VFA observed are a consequence of the 20% drop in feed intake with INF compared to CON.

As expected, both FED and INF resulted in substantial increase in total FA flows at duodenum when compared to CON. However, when the total FA flows at duodenum are expressed as a percentage of total FA intake, there was a net 35% increase in total FA flow at duodenum for cows on CON diet, a 24% decrease for cows on FED treatment, and a 14% decrease for cows on INF treatment. This is in general agreement with other reports on the net disappearance of total FA in the rumen for cows supplemented with canola fat and a net appearance of FA at the duodenum for cows on the control diet (Ferlay et al., 1993; Pantoja et al., 1996, Loor et al., 2002). For example, feeding canola oil at 3.3% (Loor et al., 2002), or 7.7% (Ferlay et al., 1993), or a blend of canola oil and tallow at 5% (Pantoja et al., 1996), was found to reduce total FA flows at duodenum, as a percentage of intake, by 1%, 22%, and 11%, respectively; whereas the duodenal FA flows for the control diets in these studies were increased by 11%, 15%, and 3%, respectively. Similarly, feeding a rumen protected source of canola fat such as canolamide at 3.3% (Loor et al., 2002), or calcium salts of canola oil at 8.4% of diet DM (Ferlay et al., 1993), reduced total FA flows at duodenum by 32% and 37%, respectively. Therefore, across studies, the net loss of total FA at duodenum is dependent on the level of inclusion of canola fat. Although the possible causes are not completely understood, the reduced duodenal FA flows, relative to intake, for cows fed supplemental fat, could be due to ruminal absorption/degradation of FA, conversion of FA to ketone bodies by the ruminal epithelium, and/or direct inhibition of excess FA available in the rumen on *de novo* FA synthesis by the rumen microbes (Doreau and Chilliard, 1997). In contrast to potentially lower contribution of microbial FA to duodenal FA with FED or INF treatments, the contribution of microbial FA to total duodenal FA flows might be greater for cows on CON diet leading to a net increase in FA flow at duodenum.

The degree of biohydrogenation of unsaturated FA in the rumen, their intestinal digestibility and subsequent absorption play a critical role in influencing the FA composition of milk. When large amounts of unsaturated C_{18} FA are fed, there is an increased production of intermediate products of rumen biohydrogenation such as *trans*-11 18:1 and *cis*-9 *trans*-11 18:2 (CLA) (Bauman et al., 2000) as demonstrated in the FED treatment in this study. Conversion of 18:1*n*-9 to *trans*-11 18:1 by the ruminal microbes

(Mosley et al., 2002) might also have contributed to the increased duodenal flows of *trans*-11 18:1. The biohydrogenation of 18:1*n*-9 and CLA to 18:0, in addition to dietary 18:0, undoubtedly contributed to the nearly 2-fold increase in 18:0 flow at the duodenum with the FED treatment.

Intestinal digestibility of FA varies with the degree of unsaturation. From an extensive review of literature, Doreau and Chilliard (1997) concluded that the digestibilities of 18:1*n*-9 and 18:2*n*-6 are higher than 18:0 and 18:3*n*-3, and that the capacity of FA absorption is much higher than 1 kg/d. Overall, total tract digestibilities for FA were similar between CON and FED except for a depression in digestibility of 18:0 and 15:0. The reduction in total tract digestibility of 18:0 with FED and INF relative to CON might be due to greater duodenal flows, and hence of lower postruminal digestibility of this saturated FA. The postruminal infusion of relatively large amounts of 18:1*n*-9 may have overwhelmed the absorptive capacity of the intestine leading to a depression in digestibility. On the contrary, with FED treatment, the biohydrogenation of 18:1*n*-9 and lower duodenal flows might have resulted in improved total tract digestibility. The absorption of C_{18} unsaturated FA, in general, were increased by both FED and INF treatments as evidenced by significant increase in plasma fatty acids concentrations of both FED and INF relative to CON. The CLA isomer, *cis*-9 *trans*-11 18:2, was detectable only in the duodenal contents and not in the feces of FED cows. It is likely that both CLA and *trans*-11 18:1 are completely digested and absorbed, rather than transformed postruminally, because the proportions of these fatty acids were increased nearly 4-fold in plasma of FED compared to CON or INF treatments.

In this study, canola oil supplementation reduced milk fat percentage through potentially different mechanisms. The reduction in milk fat percentage with FED may be attributable to a dilution of milk fat by the marginal increase in milk yield and to ruminally derived *trans*-11 18:1 (Romo et al., 2000). However, it is unlikely that *trans*-11 18:1 was responsible for the reduction in percentage and yield of milk fat with INF because neither duodenal flows (Table 4.5) nor milk concentrations of *trans*- 11 18:1 (Table 4.8) differed from CON. A similar response was observed by Chouinard et al. (1998) with feeding calcium salts of canola oil. It is more likely that the depression in

milk fat with INF is a result of inhibition of *de novo* mammary lipogenesis by long-chain FA (18:1*n*-9, 18:2*n*-6, and /or 18:3*n*-3). In contrast to our study on cows in late-lactation, duodenal infusion of a similar amount of canola oil to mid-lactation Holsteins did not affect milk fat content and yield (Gagliostro and Chilliard, 1991), suggesting that relative to cows in mid-lactation late-lactation cows might be more susceptible to milk-fat depression caused by postruminal infusion of canola oil.

Canola oil supplementation had a profound influence on milk FA composition. The reduction in 14:0 and 16:0 with INF was much greater than that reported by other researchers with abomasal infusion of canola oil (DePeters et al., 2001), but similar to the changes observed with duodenal infusion of canola oil to mid-lactation Holstein cows (Chilliard et al., 1991; Ottou et al., 1995). The greater reduction in medium chain FA with postruminal oil infusion observed in our study, compared to most other studies, may be due to additive effects of a decrease in VFA production (Table 4.4) and a direct inhibitory effect of long chain FA on *de novo* synthesis of medium chain FA in the mammary gland. However, the confounding effects of reduction in VFA production on *de novo* lipogenesis could not be separated from the direct effects of long chain FA in this study. Despite this limitation, comparisons of the magnitude of reduction of these FA between studies reveals that the reduction is not linear to the amount of oil infused postruminally. This might be due to desensitization of mammary lipogenic activity to the inhibitory effects of large amounts of long chain FA and/or effects of stage of lactation. The increase in 18:1*n*-9 : 18:0 ratio with INF relative to CON is likely due to increased supply of 18:1*n*-9 to the mammary gland. The magnitude of drop in 14:0 and 16:0 with FED in this study is greater than that reported by DePeters et al. (2001) but similar to the decrease of 14:0 and 16:0 reported by Jenkins (1999). The lack of differences between CON and FED in their impact on 18:2*n*-6 and 18:3*n*-3 concentrations are due to their extensive biohydrogenation which lead to lower availability and intestinal absorption of these FA. The increased duodenal flows and absorption of the biohydrogenation intermediate *trans*-11 18:1 might have contributed to the increased levels of this isomer in milk of cows fed canola oil.

Dietary manipulation of CLA levels in late-lactation cows has received little attention. In this study, the proportion of CLA was increased by 166% with feeding canola oil compared to the control diet. The relative increase of CLA in our study is greater than the 65% increase with feeding rapeseed to mid-lactation cows (Stanton et al., 1997), but lower than the 277% increase with feeding calcium salts of canola oil fatty acids in early-lactation cows (Chouinard et al., 2001). According to Bauman et al. (2000) the majority of this CLA isomer is likely to be synthesized through desaturation of *trans*-11 18:1 by mammary Δ^9-desaturase activity. In this study, the linearity between *trans*-11 18:1 and CLA in milk appears to be much stronger ($y = 0.221x + 0.067$, $r^2 = 0.91$, $P < 0.001$) than the relationship between these two FA in duodenal contents ($y = 0.048x - 0.007$, $r^2 = 0.76$, $P < 0.01$) suggesting that mammary Δ^9-desaturase activity might be important in CLA synthesis. Current dietary guidelines for humans are to limit the intakes of saturated FA and polyunsaturated FA to 10% and 15% of total energy intake, with the combined intakes of oleic acid and carbohydrates at 60 - 70% of energy intake (Franz et al., 2002). In this study, feeding canola oil increased the proportion of oleic acid and CLA at the expense of saturated FA in milk, which may have favorable health implications for humans.

To summarize, feeding canola oil at 1 kg/d did not exert any adverse effects on ruminal fermentation characteristics, or on ruminal and total tract digestibilities of dry matter, crude protein and fiber. Incomplete biohydrogenation of C_{18} unsaturated FA from canola oil might have resulted in increased duodenal flow of intermediates such as *trans*-11 18:1 and CLA, and their subsequent transfer to milk. In contrast to the effects of dietary canola oil, abomasal infusion of oil resulted in reductions in feed intake, total VFA production, nutrient flows to the intestine, total FA digestibility and, milk and milk fat yield. Abomasal infusion of canola oil increased concentrations of 18:1*n*-9, 18:2*n*-6 and 18:3*n*-3 and, similar to feeding oil, reduced the proportions of saturated and medium chain FA in milk.

4.4.2.Gene expression, plasma concentrations of hormones and metabolites, fatty acid

composition of plasma and adipose tissue

As discussed in the previous section, the duodenal flows of unsaturated and total FA and the ratio of unsaturated to saturated FA, were much greater with INF than CON or FED, with 18:1*n*-9 and 18:2*n*-6 together explaining 89% of the variation in DMI. We attempted to delineate the satiety signals mediating fat-induced reduction in DMI by focussing on the gut hormone–CCK, the adipose tissue derived signal - leptin and plasma fatty acids.

The current understanding of the physiology of CCK in monogastrics is that intestinal delivery of lipid stimulates the secretion of CCK from duodenal epithelial cells which then acts at CCK_A receptors to inhibit gastric emptying and (or) reduce food intake (Moran, 2000). Very few studies have been conducted in ruminants to address the role of CCK in mediating the effects of dietary fat on feed intake. We found that protecting canola from ruminal metabolism, by abomasal infusion, resulted in an increase in plasma CCK concentrations and a reduction in feed intake in agreement with some (Choi and Palmquist, 1996; Choi et al., 2000) but not all (Benson and Reynolds, 2001) reports. It is likely that the stimulation of CCK gene expression by canola oil (Figure 4.2) contributed to the increase in plasma CCK concentrations. Blockade of CCK_A receptors was found to reverse the inhibitory effects of fat on feed intake (Choi et al., 2000), attesting to a role for elevated plasma CCK concentrations in mediating fat-induced reduction in feed intake. Therefore, it is possible that the increase in plasma CCK concentrations accompanying abomasal infusion of canola oil in our study contributed to the reduction in feed intake.

In contrast to published studies on the effects of rumen-protected fat on feed intake and plasma CCK concentrations in cattle (Choi and Palmquist, 1996; Choi et al., 2000), we observed a novel increase in CCK gene expression and CCK release in the absence of any changes in feed intake when canola oil was fed in the diet. This apparent paradox was unexpected and contradicts the popular notion that an increase in plasma CCK concentrations mediates the fat-induced reduction in feed intake (Choi and Palmquist, 1996; Choi et al., 2000). It is possible that the duration of treatment was not long enough to affect feed intake. If treatments were continued for a month or longer a

potential inhibitory influence of dietary canola oil on feed intake might be expected. It is also conceivable that the relative stability of the ruminal and postruminal environment with dietary canola oil could have generated strong orexigenic signals that might override any effect of CCK in reducing feed intake. Alternatively, CCK concentrations in blood may play no role in mediating satiety in cattle. In support of this: 1) portal vein infusion of CCK, to simulate the endocrine effects of the hormone, failed to reduce feed intake in sheep despite doubling of plasma CCK concentrations (Farningham et al., 1993), 2) immunoneutralization of circulating CCK had no effect on fat-induced inhibition of food intake in rats (Reidelberger et al., 1994) and 3) no direct relationship was found between fat-induced elevation in plasma CCK concentrations and food suppression in humans (French et al., 2000). Thus, the consensus opinion appears to be that the satiety effects of CCK are mediated through paracrine or neurocrine effects on low affinity binding sites on vagal afferents, rather than through an endocrine mechanism of action (Moran, 2000).

To reconcile the divergent effects of fat supplementation on feed intake and plasma CCK concentrations in our study, we speculate that any anorexigenic effects of CCK, mediated either through an increase in plasma concentrations or locally at gastrointestinal vagal afferents, were subdued by an orexigenic drive caused by a stable ruminal and postruminal environment. On the other hand, with postruminal oil infusion, an increase in local and (or) systemic CCK concentrations could be one of the factors that inhibited gastric motility, as evidenced by reduced nutrient flows, and generated a strong satiety response. Nevertheless, the dissociation between effects on intake and CCK observed when canola oil was either fed or infused postruminally necessitates a reassessment of the popular notion that fat-induced increase in plasma CCK concentrations mediate the reduction in feed intake in cattle.

The synergistic interaction between CCK and leptin in suppression of food intake in rodents is well documented (Wang et al., 2000). Further, the possibility of a positive-feedback loop between CCK and leptin is revealed by the fact that exogenous CCK caused a rapid release of gastric leptin into circulation (Bado et al., 1998), and administration of leptin increased plasma CCK levels (Guilmeau et al., 2002) in rats. In view of such synergism in rodent models, our interest was in testing whether such an

interaction exists in ruminants. Under our experimental conditions, fat supplementation had no significant effect on plasma leptin concentrations or on the expression of the leptin gene in adipose tissue. Further, in contrast to studies in rodents reporting the presence of leptin mRNA in gastric mucosa (Bado et al., 1998), and the existence of CCK_B receptor transcripts in adipose tissue (Attoub et al., 1999), we were unable to detect transcripts for leptin in the gastrointestinal tract (Chelikani et al., 2003) and for CCK_B receptor in adipose tissue (unpublished data) using an RT-PCR based approach. Therefore, we are skeptical about the existence of a positive feed back loop between gastric leptin and CCK, and between CCK and adipose tissue leptin production in cattle. However, the presence of transcripts for the long-form of the leptin receptor along the gastrointestinal tract of calves (Chelikani et al., 2003) indicates that leptin may be involved in lipid absorption as has been suggested for rodents (Morton et al., 1998). In this study, neither leptin secretion nor energy balance were significantly affected by treatments. Given the relative stability of leptin gene expression and plasma leptin concentrations in this study, it is unlikely that leptin is involved in mediating the inhibitory effects of canola oil on feed intake in cattle. Future research should determine whether the response of plasma leptin concentrations to fat supplementation is dependent on the duration of supplementation, degree of unsaturation of fat, stage of lactation and (or) body condition.

The influence of canola fat supplementation on the circulating concentrations of insulin has been contradictory, with some studies reporting an increase (Khorasani and Kennelly, 1998), a decrease (Benson and Reynolds, 2001), or no effect (Delbecchi et al., 2001; DeLuca and Jenkins, 2000). From an extensive review of literature Staples et al. (1998) concluded that the effects of fat supplements on the circulating concentrations of insulin are dependent on the energy status of the animals; insulin concentrations are increased with an improvement in energy intake and decreased with a reduction in intake. We did not observe any significant differences in plasma insulin concentrations in our study, although the concentrations were numerically higher with feeding and lower with infusion. In agreement with DeLuca and Jenkins (2000) canola supplementation had no effect on the levels of plasma IGF-I in this study.

The importance of plasma FA in mediating the hypophagic effects of fat supplements in cattle seems to have been overlooked. A role for oxidation of NEFA in the regulation of feed intake has been proposed, but there is no conclusive evidence to support this theory (Choi et al., 1997). On the other hand, there is convincing evidence to suggest that circulating fatty acids, especially 18:1*n*-9 and 18:2*n*-6, could be directly involved in intake regulation. In rodents, plasma 18:1*n*-9 (Sztriha and Betz, 1991) and 18:2*n*-6 (Edmond et al., 1998) were found to rapidly cross the blood-brain barrier and centrally administered 18:1*n*-9 acid was found to inhibit food intake and reduce neuropeptide-Y independent of any changes in leptin (Obici et al., 2002). Further, intestinal infusions of 18:1*n*-9 (Covasa et al., 2000) and 18:2*n*-6 (Phifer and Berthoud, 1998) were reported to increase c-Fos expression in intestinal myenteric plexus (Covasa et al., 2000), in the nucleus of the solitary tract and in the area postrema of the hindbrain (Covasa et al., 2000; Phifer and Berthoud, 1998). In our study, with abomasal infusion of canola oil, the proportions of 18:1*n*-9 and 18:2*n*-6 in plasma were significantly greater than those observed in the control or feeding treatments. With feeding oil, the 20% increase in plasma 18:1*n*-9 with feeding may not have reached a threshold level to exert an adverse affect on feed intake. Extrapolating from rodent studies, the reduction in feed intake with abomasal infusion in our study could be a consequence of the effect of elevated plasma 18:1*n*-9 and (or) 18:2*n*-6 concentrations on vagal afferents to inhibit gastric emptying via a vago-vagal reflex, and (or) direct affects within the hypothalamus. It is apparent from our data that the hypophagic effects of fat in dairy cattle are not mediated through CCK alone, or through oleic and linoleic acids, and attest to the multiplicity and redundancy of the satiety system in cattle.

Compared to the plethora of literature documenting the effects of fat supplementation on milk fatty acid composition, it seems that the topic of whether dietary fat supplementation modifies the fatty acid composition of adipose tissue of dairy cattle has received little attention. The proportions of 16:0 and 16:1 were reported to be reduced in subcutaneous adipose tissue of beef cattle fed extruded full-fat canola seed (Rule et al., 1994). Similarly, we also observed a reduction in the proportions of 16:0 and 16:1 in adipose tissue; moreover, the reduction was greater with infusion than feeding.

As expected, the proportion of *cis*-18:1*n*-9 was increased with fat supplementation and was markedly elevated with abomasal infusion. Despite these changes we were unable to detect treatment differences in the transcript abundance for LPL and ACC in adipose tissue. In contrast to our data, duodenal infusion of canola oil had no effect on *cis*-18:1*n*-9 concentrations, increased 18:2*n*-6 and 18:2*n*-3 concentrations, and increased LPL activity, in perirenal adipose tissue of Holstein cows in mid-lactation (Chilliard et al., 1991). The duration of fat supplementation in our study was 16-d in each period compared to approximately 42-d periods in the study of Chilliard et al. (1991). Therefore, it is likely that the duration of infusion or feeding in our study is relatively short to have a significant impact on polyunsaturated FA content in adipose tissue. Further differences in the stage of lactation, and adipose tissue site (perirenal vs subcutaneous), might also explain the discrepancies between studies. It is also possible that ACC and LPL enzymes are regulated at the post-transcriptional level in the short-term similar to the time-line in our study. The lack of changes in abundance of leptin transcripts in the presence of increased *cis*-18:1*n*-9 in adipose tissue indicates that oleic acid may not be involved in regulation of leptin in cattle in the short-term.

In summary, both feeding and abomasal infusion of canola oil resulted in a similar increase in gene expression and plasma concentrations of CCK but only infusion caused a reduction in feed intake. Our data do not support the existence of an endocrine mechanism of action for CCK in mediating satiety. Rather, our results are consistent with a mechanism whereby the hormone acts either through paracrine and (or) neurocrine routes to influence feed intake in cattle. Plasma concentrations of leptin, insulin and IGF-I were not altered with fat supplementation, indicating that these signalling molecules may not be involved in mediating the short-term effects of dietary fat on feed intake. It is more likely that the elevation in plasma concentrations of oleic and (or) linoleic acids mediate the hypophagic effects of fat in synergy with hormonal signals such as CCK.

Table 4.1. Ingredient and chemical composition of basal diet.

Item	
Ingredient (gm/100gm DM)	
Alfalfa silage	19.9
Barley silage	19.9
Grass hay	9.9
Rolled barley	30.22
Canola meal	8.55
Soybean meal	3.30
Dried distillers	6.48
Mineral-Vitamin mix†	1.49
Chemical composition	Mean ± SE
DM (gm/100gm fresh weight)	49.93 ± 5.11
OM (gm/100gm fresh weight)	88.28 ± 2.44
CP (gm/100 gm DM)	18.42 ± 2.63
Crude fat (gm/100gm DM)	2.59 ± 0.57
NDF (gm/100gm DM)	36.72 ± 3.75
ADF (gm/100gm DM)	23.32 ± 1.72
Ash (gm/100gm DM)	1.49 ± 0.58
GE (Mcal/kg DM)	4.83 ± 1.03

† Contained (/kg DM) 125 g Na, 97 g Cl, 103 g Ca, 80 g P, 86 g Mg, 80 mg Co, 400 mg Cu, 200 mg I, 6000 mg Mn, 400 mg Se, 800 mg Zn, 3,846 KIU Vitamin A, 2,292 KIU Vitamin D, and 6,539 IU Vitamin E (Champion Feed Services Ltd., Edmonton, Canada).

Table 4.2. Fatty acid composition of canola oil.

Fatty acid methyl esters (FAME: gm/100gm)	Mean ± SE
14:0	0.04 ± 0.01
15:0	0.04 ± 0.01
16:0	4.21 ± 0.03
16:1	0.27 ± 0.02
18:0	2.08 ± 0.03
cis-18:1*n*-9	62.35 ± 0.14
18:2*n*-6	18.51 ± 0.06
18:3*n*-3	8.97 ± 0.36
Others	2.79 ± 0.06

Table 4.3. Effect of feeding or abomasal infusion of canola oil on ruminal and total tract digestibilities of dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent (NDF) and acid detergent fiber (ADF) fibers, and cellulose. Nutrient intake and duodenal flows are in kg/d; ruminal and total tract digestibilities are expressed as gm/100gm.

	Control	Feeding	Infusion	SEM
DM				
Intake	14.02[a]	14.54[a]	11.33[b]	0.66
Duodenal flow	9.43[a]	9.60[a]	6.34[b]	1.23
Ruminal digestibility	42.25	45.08	36.45	4.62
Total tract digestibility	62.26	61.11	57.45	2.54
OM				
Intake	12.19[a]	12.38[a]	10.10[b]	0.53
Duodenal flow	7.83[a]	8.08[a]	5.22[b]	1.09
Ruminal digestibility	45.37	48.47	41.77	5.43
Total tract digestibility	66.86	62.76	60.41	2.83
CP				
Intake	2.66[ab]	3.23[a]	2.13[b]	0.27
Duodenal flow	2.16[a]	1.95[ab]	1.27[b]	0.31
Ruminal digestibility	24.17[a]	37.19[b]	26.41[ab]	1.98
Total tract digestibility	66.78	69.13	64.55	3.98
NDF				
Intake	7.40[a]	8.39[a]	6.08[b]	0.39
Duodenal flow	5.57	5.54	3.64	0.70
Ruminal digestibility	43.29	50.66	41.78	6.59
Total tract digestibility	49.39	44.77	45.13	3.22
ADF				
Intake	4.29[a]	4.56[a]	3.36[b]	0.18
Duodenal flow	3.68	3.53	2.39	0.47

Ruminal digestibility	34.69	44.29	31.26	6.09
Total tract digestibility	42.61	36.61	37.31	4.27
Cellulose				
Intake	3.16[a]	3.38[a]	2.48[b]	0.16
Duodenal flow	1.95	1.90	1.19	0.25
Ruminal digestibility	53.13	60.35	54.18	5.76
Total tract digestibility	57.76	54.18	55.49	2.84

[a, b] Means with different superscripts within the same row differ ($P < 0.05$).

Table 4.4. Effect of feeding or abomasal infusion of canola oil on ruminal pH, ammonia nitrogen (NH_3N), total concentrations (m*M*) and proportions of volatile fatty acids (VFA).

	Control	Feeding	Infusion	SEM
pH	6.29	6.23	6.43	0.06
NH_3N (mg/dl)	15.23	16.14	16.22	1.83
Total VFA (m*M*)	107.26^{a}	105.33^{a}	93.94^{b}	2.19
Individual VFA (mol/100 mol)				
Acetate (A)	65.71	65.60	59.67	3.36
Propionate (P)	23.77^{a}	20.63^{ab}	18.06^{b}	2.25
Butyrate	12.41^{ab}	14.04^{a}	11.24^{b}	0.65
Isobutyrate	1.18	1.11	1.18	0.05
Valerate	2.15	1.69	1.50	0.14
Isovalerate	1.67	1.80	1.88	0.09
A : P ratio	3.11	3.23	3.35	0.11
BCFA†	1.43	1.45	1.53	0.06

[a, b] Means with different superscripts within the same row differ ($P < 0.05$).

† Branched chain fatty acids = isobutyrate + isovalerate.

Table 4.5. Effect of feeding or abomasal infusion of canola oil on intake and duodenal flow of fatty acids.

	Control	Feeding	Infusion†	SEM
Intake (g/day)				
14:0	0.14[a]	2.65[b]	2.40[b]	0.10
15:0	3.12	4.39	4.77	0.92
16:0	43.96[a]	97.60[b]	89.48[b]	3.28
16:1	0.28[a]	3.82[b]	2.82[b]	0.69
18:0	75.06[a]	122.44[b]	112.07[b]	7.63
cis-18:1*n*-9	76.45[a]	620.59[b]	630.79[b]	41.33
18:2*n*-6	38.47[a]	195.38[b]	201.55[b]	15.89
18:3*n*-3	6.75[a]	100.80[b]	89.99[b]	5.99
Others	0.65[a]	3.36[a]	7.64[b]	1.37
Total	244.19[a]	1154.29[b]	1138.52[b]	62.52
Duodenal flow (g/day)				
14:0	3.19	3.38	3.87	0.39
14:1	3.44	3.62	4.87	1.22
15:0	2.39	2.70	3.15	0.74
16:0	58.71[a]	104.19[b]	99.76[b]	7.73
16:1	6.54	6.74	6.96	1.46
18:0	175.05[a]	440.68[b]	278.01[c]	37.60
trans-11 18:1	11.51[a]	53.77[b]	13.02[a]	5.71

cis-18:1*n*-9	45.61[a]	241.63[b]	569.83[c]	92.88
18:2*n*-6	13.54[a]	16.94[a]	83.49[b]	8.21
cis-9 *trans*-11 18:2 (CLA)	ND‡	3.09	ND‡	0.51
18:3*n*-3	3.86[a]	2.32[a]	36.01[b]	3.53
Others	11.38	13.11	21.68	5.81
Total fatty acids	328.74[a]	880.41[b]	973.48[b]	88.92
Total saturated§	238.53[a]	529.71[b]	329.90[a]	46.24
Total unsaturated¶	88.87[a]	299.32[b]	599.03[c]	55.89
Unsaturated : Saturated	0.66[a]	0.65[a]	2.71[b]	0.45

[a, b, c] Means with different superscripts within the same row differ ($P < 0.05$).

† Intake and duodenal flows include values for the infused oil

‡ Not detectable

§ 14:0, 15:0, 16:0, 18:0.

¶ 14:1, 16:1, 18:1, 18:2, 18:3.

Table 4.6. Effect of feeding or abomasal infusion of canola oil on total tract digestibility and postruminal absorption of fatty acids (FA).

	Control	Feeding	Infusion	SEM
Digestibility (g/100 gm)				
14:0	73.99	93.86	65.02	14.89
15:0	94.13[a]	73.34[b]	72.61[b]	6.49
16:0	92.85[a]	87.93[a]	71.44[b]	6.03
18:0	85.19[a]	70.93[b]	69.71[b]	2.99
cis-18:1*n*-9	97.59[a]	95.87[a]	78.72[b]	1.73
18:2*n*-6	98.91	98.86	98.06	0.60
18:3*n*-3	98.88	98.15	96.51	1.21
Total FA	84.84[a]	91.09[a]	76.69[b]	6.06
Absorption (g/day)				
14:0	2.83	3.16	2.24	0.66
15:0	2.29	2.25	2.12	0.90
16:0	53.59[a]	89.45[b]	81.05[b]	7.18
18:0	145.12[a]	314.20[b]	173.87[c]	17.79
cis-18:1*n*-9	39.20[a]	231.01[b]	438.32[c]	29.58
18:2*n*-6	12.87[a]	10.14[a]	82.23[b]	4.06
18:3*n*-3	3.10[a]	1.73[a]	35.45[b]	1.27
Total FA	265.98[a]	764.37[b]	867.45[b]	90.03

[a, b, c] Means with different superscripts within the same row differ ($P < 0.05$).

Table 4.7. Effect of feeding or abomasal infusion of canola oil on milk composition and yield.

	Control	Feeding	Infusion	SEM
Milk yield (kg/d)	11.35	14.05	12.37	0.89
Composition (%)				
Fat	3.39[a]	2.58[b]	2.03[b]	0.23
Protein	2.99	3.19	3.21	0.09
Lactose	4.08	4.28	4.02	0.14
Production (kg/d)				
Fat	0.39[a]	0.37[a]	0.25[b]	0.02
Protein	0.33	0.45	0.39	0.03
Lactose	0.46	0.60	0.49	0.04

[a, b] Means with different superscripts within the same row differ ($P < 0.05$).

Table 4.8. Effect of feeding or abomasal infusion of canola oil on milk fatty acid composition.

	Control	Feeding	Infusion	SEM
	gm/100 gm FAME			
4:0 – 10:0	6.26	5.11	5.59	0.43
11:0	0.29^{a}	0.19^{ab}	0.12^{b}	0.03
12:0	3.63	2.82	3.68	0.57
12:1	0.19^{a}	0.08^{b}	0.02^{b}	0.03
14:0	11.94^{a}	9.77^{b}	8.07^{c}	0.45
14:1	0.88^{a}	0.84^{a}	0.41^{b}	0.07
15:0	1.87^{a}	1.11^{b}	0.79^{b}	0.18
15:1	0.22	0.29	0.15	0.09
16:0	30.12^{a}	21.19^{b}	18.33^{c}	0.55
16:1	1.62^{a}	1.29^{a}	0.86^{b}	0.11
17:0	0.86^{a}	0.56^{b}	0.47^{c}	0.03
17:1	0.29	0.13	0.15	0.05
18:0	10.65^{a}	13.76^{b}	8.75^{c}	0.59
trans-11 18:1	1.95^{a}	5.74^{b}	2.15^{a}	0.36
cis-18:1*n*-6	0.68^{a}	1.54^{b}	0.48^{c}	0.05
cis-18:1*n*-9	24.2^{a}	30.53^{b}	34.62^{c}	0.92
cis-18:1*n*-11	0.71^{a}	0.86^{a}	2.20^{b}	0.16
Total 18:1	27.56^{a}	38.67^{b}	39.46^{b}	0.88

18:2*n*-6	2.37[a]	1.87[a]	8.69[b]	0.29
cis-9 *trans*-11 18:2 (CLA)	0.51[a]	1.36[b]	0.51[a]	0.03
18:3*n*-3	0.42[a]	0.38[a]	3.36[b]	0.16
20:0	0.13[a]	0.22[b]	0.06[a]	0.03
20:1	0.15[a]	0.33[b]	0.53[c]	0.05
18:1 : 18:0	2.64[a]	2.85[a]	4.61[b]	0.13
Saturated†	62.42[a]	52.61[b]	43.36[c]	0.95
Monounsaturated‡	30.76[a]	41.65[b]	41.56[b]	0.93
Polyunsaturated§	3.89[a]	3.56[a]	12.64[b]	0.40
Medium-chain¶	51.95[a]	38.29[b]	33.05[c]	1.31
Long-chain††	41.79[a]	56.59[b]	61.35[b]	1.55

[a, b, c] Means with different superscripts within the same row differ ($P < 0.05$).

† 4:0, 6:0, 8:0, 10:0, 11:0, 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0.

‡ 12:1, 14:1, 15:1, 16:1, 17:1, 18:1, 20:1.

§ 18:2, 18:3.

¶ 11:0, 12:0, 12:1, 14:0, 14:1, 15:0, 15:1, 16:0, 16:1, 17:0, 17:1.

†† 18:0, 18:1, 18:2, 18:3, 20:0, 20:1.

Table 4.9. Primer conditions for semi-quantitative RT-PCR amplification of each target gene. Forward (F) and reverse (R) primer pairs for each target gene, annealing temperature (AT), length of PCR product (base pairs, bp), linear range of amplification and number of cycles selected (in parentheses), and the GenBank accession number from which the primer pairs were designed are shown.

Target†	Primers[1]	AT (^{0}C)	Length (bp)	Linear range	Accession no:
Leptin	F: 5'-GTGCCCATCCGCAAGGTCC-3'	60^0 C	441	24 – 32 (28)	U43943
	R: 5'-TCAGCACCCGGGACTGAGG-3'				
LPL	F: 5'-AGGACACTTGCCACCTCATTCC-3'	62^0 C	682	22 – 34 (26)	GI4768902
	R: 5'-GAGTCAATGAAGAAGAGATGAACGG-3'				
ACC	F: 5'-ATAGACAGAGTCATCGAGTTTGTGC-3'	60^0 C	285	30 – 38 (34)	GI6006404
	R: 5'-TGCTGGATAATCTTGGCTTCAGAATC-3'				
GAPDH	F: 5'-CTGGCAAAGTGGACATTGTCGCC-3'	65^0 C	572	20 – 28 (25)	GI285902
	R: 5'-CTTGGCAGCGCCGGTAGAAGC-3'				
CCK	F: 5'-TCGGGCCGAATGTCTGTG-3'	57^0 C	420	26 – 36 (30)	Submitted to GenBank
	R: 5'-TTACGGAACAGAAGAAAATCACTTTA-3'				

† Lipoprotein lipase (LPL), Acetyl-coA carboxylase (ACC), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Cholecystokinin (CCK).

Table 4.10. Effect of feeding or abomasal infusion of canola oil on plasma glucose and plasma fatty acid composition.

Plasma variables	Control	Feeding	Infusion	SEM
Glucose (mg/dl)	80.69	72.04	76.25	5.11
Fatty acid methyl esters (gm/100 gm)				
10:0	0.83[a]	0.58[b]	0.18[b]	0.02
12:0	0.98[a]	0.18[b]	0.19[b]	0.38
14:0	0.62[a]	0.42[b]	0.12[b]	0.10
15:0	0.20	0.15	0.21	0.10
16:0	12.62[a]	12.51[a]	6.49[b]	1.52
16:1	2.14	1.51	2.70	1.09
17:1	1.98[a]	1.89[a]	0.32[b]	0.18
18:0	16.89[a]	25.09[b]	17.16[c]	2.54
trans-11 18:1	0.47[a]	2.55[b]	0.30[a]	0.24
cis-18:1*n*-6	1.45	1.69	1.03	0.27
cis-18:1*n*-9	10.09[a]	12.39[b]	17.19[c]	1.56
cis-18:1*n*-11	0.09[a]	0.10[a]	0.14[b]	0.03
18:2*n*-6	46.98[a]	38.61[b]	48.02[c]	0.44
cis-9 *trans*-11 18:2 (CLA)	0.02[a]	0.08[b]	0.02[a]	0.01
18:3*n*-3	3.83[a]	2.74[b]	3.88[a]	0.55
Total (μg/ml)	1974.4[a]	3590.2[b]	3750.2[b]	266.4

[a, b, c] Means with different superscripts within the same row differ ($P < 0.05$).

Table 4.11. Effect of feeding or abomasal infusion of canola oil on fatty acid composition of subcutaneous adipose tissue.

Fatty acid methyl esters (gm/100 gm)	Control	Feeding	Infusion	SEM
14:0	2.79	3.01	3.01	0.16
14:1	0.69	0.53	0.48	0.10
15:0	0.42	0.45	0.51	0.05
16:0	26.67[a]	25.07[b]	23.41[c]	0.28
16:1	4.67[a]	4.19[b]	3.86[c]	0.19
18:0	17.78	18.04	18.40	1.12
trans-11 18:1	1.93	2.07	2.26	0.16
cis-18:1*n*-6	2.83	3.03	3.22	0.20
cis-18:1*n*-9	36.60[a]	38.14[a]	40.92[b]	1.02
cis-18:1*n*-11	1.93	2.03	2.22	0.64
18:2*n*-6	1.92	1.86	1.72	0.16
cis-9 *trans*-11 18:2 (CLA)	0.40	0.46	0.38	0.08
18:3*n*-3	0.27	0.22	0.21	0.04

[a, b, c] Means with different superscripts within the same row differ ($P < 0.05$).

Figure 4.1. Effect of feeding or abomasal infusion of canola oil on temporal changes in plasma concentrations of cholecystokinin (CCK; A), leptin (B), insulin (C), and IGF-1 (D). Treatment differences at each time point are indicated by letters (a, b, c $P < 0.05$).

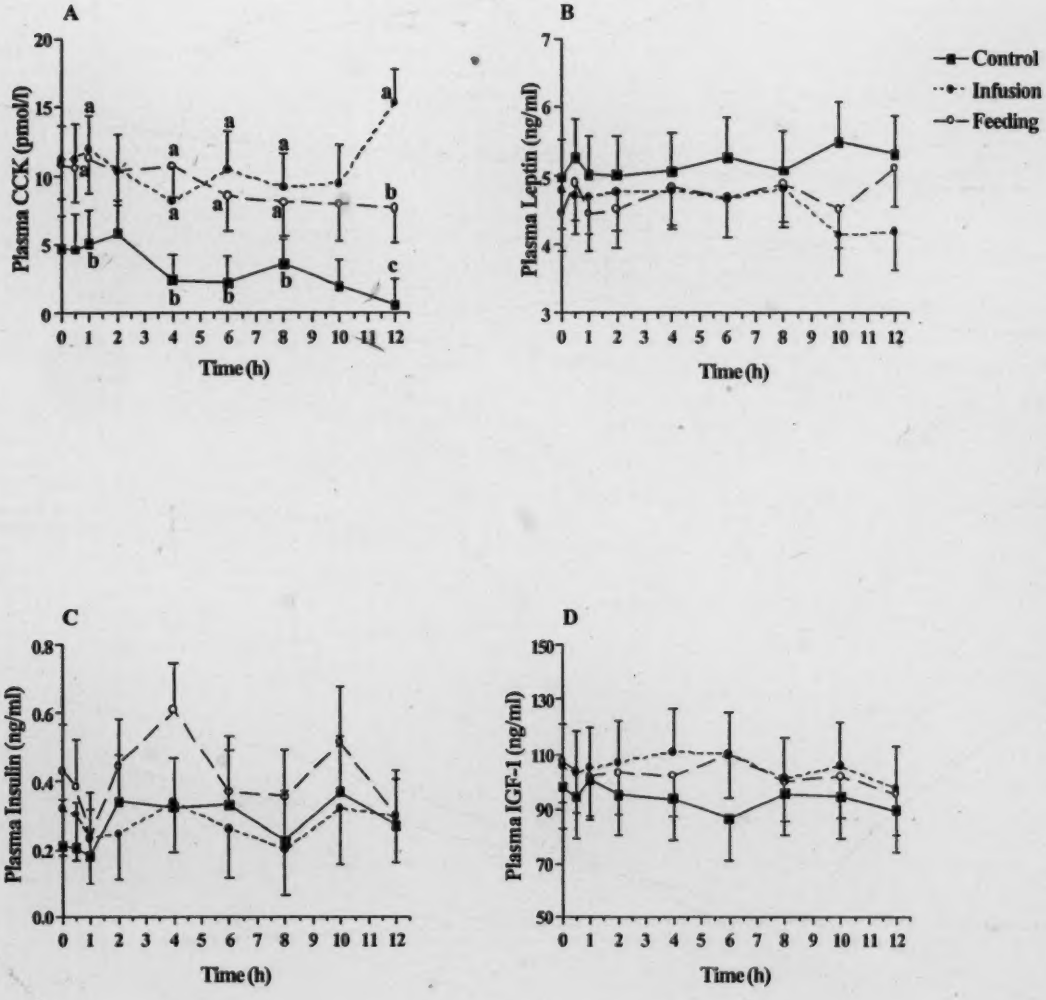
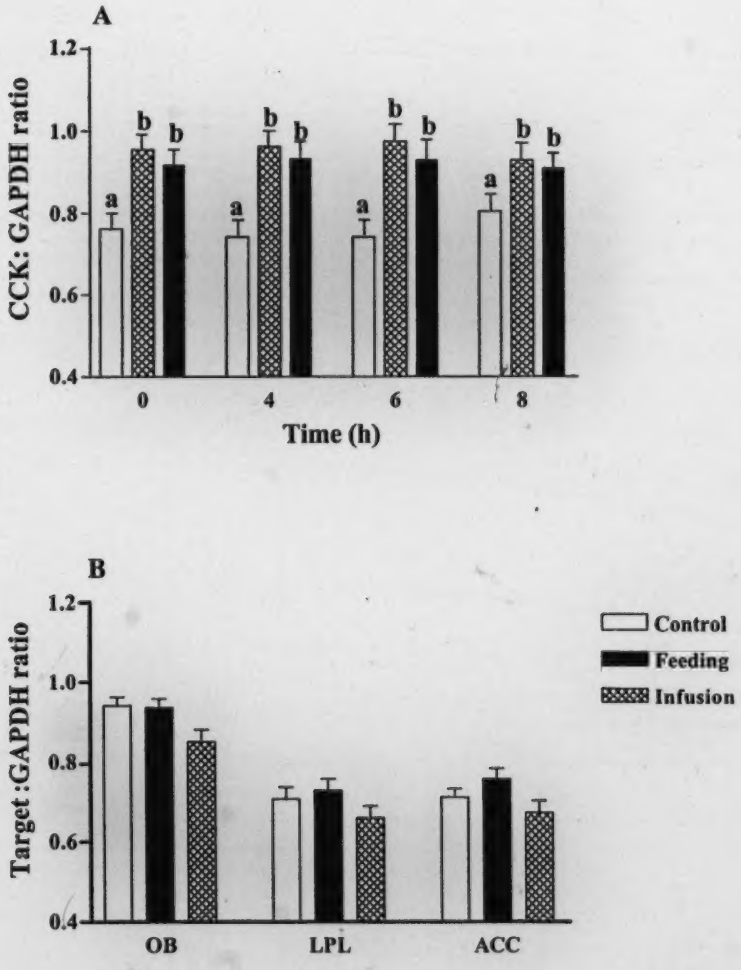


Figure 4.2. Effect of feeding or abomasal infusion of canola on mRNA abundance of cholecytokinin (CCK) and adipose tissue genes analyzed by semi-quantitative reverse RT-PCR. Temporal changes in CCK : GAPDH mRNA ratio in duodenal biopsy samples are shown (A). The ratios of mRNA abundance of adipose tissue genes (leptin, (OB), lipoprotein lipase (LPL), and acetyl-coA carboxylase (ACC)) to GAPDH are also presented (B). Treatment differences are indicated by letters (a, b $P < 0.05$).



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CHAPTER 5

Effect of isoenergetic jugular infusion of lipid or glucose on plasma concentrations of leptin and other hormones and metabolites in early and late- lactation dairy cows[1]

5.1. Introduction

Leptin, a hormone secreted primarily from adipose tissue, has been shown to play an important role in regulating feed intake in both ruminants and monogastrics (Ingvartsen and Boisclair, 2001), and in influencing carbohydrate and lipid metabolism in monogastric species (Baile et al., 2000). In the long term, circulating leptin concentrations are strongly associated with the degree of adiposity and the plane of nutrition (Delavaud et al., 2000). In the short-term, plasma leptin concentrations are reported to be reduced with fasting in beef cattle (Amstalden et al., 2000; Amstalden et al., 2002) and sheep (Marie et al., 2001, Daniel et al., 2002) independent of changes in body weight. As demonstrated in Chapter 3 the response of plasma leptin concentrations to fasting is dependent on the physiological stage of the animal; leptin levels were sharply reduced with fasting in early lactating dairy cows, but these responses were blunted in non-lactating dry cows and postpubertal heifers. We also found a positive correlation of leptin with glucose, insulin, and insulin-like growth factor-1 (IGF-1), and negative correlation with nonesterified fatty acids (NEFA), suggesting that these plasma variables might play a role in the short-term regulation of leptin in dairy cattle. However, the role of specific macronutrients in the short-term regulation of leptin secretion in ruminants has received little attention.

The stimulatory effect of dietary carbohydrate and fat on leptin secretion in rodents is well documented (Coleman and Hermann, 1999). As described in Chapter 4 feeding or abomasal infusion of canola oil to dairy cattle had no significant affect on gene expression and plasma concentrations of leptin. In that study the animals were relatively thin-conditioned, in late lactation, and the body weight and body condition score were not

[1] A version of this chapter has been submitted to the Journal of Nutrition

affected by treatments. If the leptin response to fat supplementation is dependent on body fat reserves it may be reasoned that cows in good body condition will exhibit a robust response compared to thin conditioned animals.

As part of the homeorhetic adaptations which occur in cows during early lactation, the adipose tissue develops resistance to insulin mediated glucose and acetate uptake, and concomitantly becomes sensitized to the lipolytic effects of elevated concentrations of plasma growth hormone (GH) (Bell and Bauman, 1997). An inhibitory effect of GH on leptin gene expression in bovine adipose tissue explants has also been reported recently (Houseknecht et al., 2000). Based on these observations it was hypothesized that plasma leptin concentrations would exhibit a strong response to energy supplementation in late lactation cows with adequate fat reserves compared to thin conditioned cows in early lactation. To test this hypothesis, and to minimize potential metabolism of energy supplements in the gastrointestinal tract, the effects of parenteral infusions of glucose and lipid were compared in this study. The specific objectives were to determine the effects of isoenergetic jugular infusions of dextrose and lipid to cows in early and late lactation on dry matter intake (DMI), milk yield and composition, and plasma concentrations of leptin, insulin, insulin-like growth factor-1 (IGF-1), GH, glucose and nonesterified fatty acids (NEFA).

5.2. Materials and methods

5.2.1. Cows, experimental design, and treatments

The experiments were conducted at the Dairy Research and Technology Centre, University of Alberta, with all animal procedures approved by the University of Alberta Animal Policy and Welfare Committee (protocol number 2000 - 75C). Experiments were performed between March and October 2001, when the cows were in early lactation (DIM 32 ± 3) and again in late lactation (DIM 249 ± 3). The six multiparous cows used in this study were housed in tie-stalls, fed at about 0900 h to ensure 5% orts and had free access to water at all times. To minimize the potential confounding effects of diets on hormonal and metabolite response to infusion at different stages of lactation, the animals were fed, over a 10-day (d) adaptation period, a common total mixed ration (TMR)

during both stages of lactation (Table 5.1).

The experiment was designed as a replicated 3 x 3 Latin square design with three treatments, three experimental periods each of 2 d, and an intervening 2 d period to minimize carry-over effects of previous treatments. All parenteral solutions used in this study were sterile and were from Baxter® Corporation (Mississauga, ON, Canada). The treatments consisted of intrajugular infusions of 2 L of either 1) Saline- control (CON), 2) Glucose (GLU, 50% Dextrose®, 1.7 kcal/ml), and 3) Lipid (LIP, 20% Intralipid®, 2 kcal/ml). According to the manufacturer, Intralipid is an aqueous emulsion of 20% soybean oil (containing 50% linoleic acid, 26% oleic acid, 10% palmitic acid, 8% linolenic acid, and 3.5% stearic acid), 2.25% glycerin, and 1.25% egg yolk phospholipids. It was not the intent of this study to compare the effects of hyperglycemic or hyperinsulinemic clamps on hormonal and metabolite responses at varying stages of lactation. Rather, the primary aim was to compare the responses to isoenergetic parenteral infusions of glucose or lipid. The amount of Dextrose® (~1.2 kg) and Intralipid® (~400 gm soyabean oil) infused in this study was calculated to be isocaloric and provide approximately 4 Mcal. The day before initiation of the experiment, the animals were fitted with BD Angiocath™ catheters (Cat # 382269, 2.1 mm x 133 cm, Becton Dickinson, Franklin Lakes, NJ, USA) into both jugular veins, one for infusion of solutions and the other for blood sampling. Patency of the catheters was maintained overnight with 200 IU/ml of heparinized saline. The solutions were continuously infused for 6 h from 0900 to 1500 h for two consecutive days in each period by means of a Masterflex® peristaltic pump (Labcor Inc., Anjou, QC, Canada).

5.2.2. Measurements and sampling

The amount of feed consumed was determined daily. Samples of TMR, ingredients and orts were collected on both days of each period and composited by cow within period. At the end of each period, body weights were recorded and body condition score (BCS) assessed by three individuals. Animal health was monitored frequently by checking body temperature, heart rate, and urinary ketones (Clini-2K

Reagent strips, URI-Quick®, Stanbio, USA). The cows were milked twice daily between 0400 and 0600 h, and between 1600 and 1800 h, milk yield recorded, and samples were taken for milk fat, protein and lactose analyses at the Alberta Central Milk Testing Laboratory (Edmonton, AB, Canada).

Relative to the beginning of infusions (~ 0900 h), blood samples were collected at –1, -0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10 12, and 24 h on the second day of each period. Samples were collected on ice in 10 ml vacutainer tubes (Becton Dickinson, USA) containing EDTA or sodium heparin. Prior to collecting blood samples, the catheters were flushed with 10 to 20 ml of heparinized sterile saline (20 IU/ml). Within 2 h of sampling, plasma was separated by centrifugation (1500 x g) and stored at -20^0 C until analyses for hormones and metabolites.

5.2.3. Chemical analyses

Samples of feed, ingredients and orts were dried at 60^0C for 72-h, and ground through a 1-mm screen (Thomas-Wiley laboratory mill model 4, PA, USA). Dry matter was determined by drying samples at 110^0C overnight, and OM was calculated as weight loss upon ashing for at least 6-h at 500^0C. Samples were analyzed for CP (6.25 x N; Leco FP-428 nitrogen determinator, Leco® Corporation, MI, USA), NDF, ADF and lignin (Ankom filter bag technique®, Ankom Company, NY, USA).

5.2.4. Hormone and metabolite assays

Plasma concentrations of leptin, insulin, insulin-like growth factor-1 (IGF-1), growth hormone (GH), glucose, nonesterified fatty acids (NEFA) were determined as described in Chapter 3. Intra-assay coefficient of variation (%) for leptin, insulin, IGF-1, GH, glucose and NEFA were 3.1, 5.1, 6.6, 2.7, 3.1, and 5.1, respectively.

Plasma β-hydroxybutyrate (BHBA) concentrations were determined using enzymatic assay kits (Sigma Chemical Co., St. Louis, MO, USA; procedures 310-UV). BHBA analyses were done only on samples collected at 0, 3 6, and 12 h since the beginning of infusion. The volumes of reagents and samples were modified to allow the

assays to be conducted in 96-well ELISA plates and absorbance measured with a SPECTRAmax®190 Microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA). The intra- and inter-assay coefficient of variation were 1.4% and 2.7%, respectively.

5.2.5. Statistical analyses.

Data were analyzed by ANOVA for a replicated Latin square design using the MIXED procedure of SAS (version 8.2, SAS Institute, Cary, NC). Repeated measures on plasma hormones and metabolites, and other performance parameters, were analyzed using the following univariate linear mixed model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \delta_l + \theta_m + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\alpha\delta)_{il} + (\alpha\theta)_{im} + (\alpha\beta\delta)_{ijl} + (\alpha\gamma\delta)_{ikl} + e_{ijklm}$$

where μ is the population mean, α_i is a population parameter corresponding to treatment i, β_j is a parameter corresponding to period j, γ_k is a parameter corresponding to time k, δ_l is a parameter corresponding to lactational stage l, θ_m a parameter corresponding to square m, $(\alpha\beta)_{ij}$ is the interaction of treatment and period, $(\alpha\gamma)_{ik}$ is the of treatment and time, $(\alpha\delta)_{il}$ is the interaction of treatment and stage, $(\alpha\theta)_{im}$ is the interaction of treatment and square, $(\alpha\beta\delta)_{ijl}$ is the interaction of treatment, period and stage, $(\alpha\gamma\delta)_{ikl}$ is the interaction of treatment, time and stage, and e_{ijklm} is the residual error. The Kenward-Roger procedure was used for approximating the degrees of freedom. Animal nested in treatment, period and square was considered as the subject on which repeated measures were taken and covariance structures modeled. Based on the smallest values of fit statistics for AIC, AICC and BIC criteria, the covariance structure of the unequally spaced repeated measurements for each variable was modeled either as compound symmetry, heterogenous compound symmetry, first-order antedependence, or spatial power law (Littell et al., 2000). Blood samples collected at 1 and 0.5 h before the beginning of infusion were averaged and considered as the baseline before analysis. The total area under the curve (AUC) for the profiles of hormones and metabolites were calculated by using the trapezoidal rule. Discrete data on feed intake, milk production and composition, and AUC, were analyzed by a similar model as described above except

that time and interactions with time were excluded. One cow in early lactation developed signs of severe ketosis and was removed from the trial; the data from this animal was not considered for analysis. Preplanned treatment comparisons among individual time points were made with the PDIFF option. Comparisons with P<0.01 were declared highly significant, $P < 0.05$ significant, and $0.05 < P < 0.10$ were considered as trends.

5.3. Results

5.3.1. Intake and production.

The effect of jugular infusions of Saline (CON), Dextrose (GLU) or Intralipid (LIP) on feed intake and production performance of cows in early and late lactation is shown in Table 5.2. In general, cows in late lactation had lower DMI and yields of milk and milk components, but greater BW, energy balance, and BCS compared to early lactation cows (Table 5.2; $P < 0.05$). There was a tendency ($P = 0.06$) for treatment x stage interaction to be significant for DMI; treatments had a significant influence on DMI only in late ($P < 0.05$) but not in early ($P > 0.10$) lactation. In late lactation cows, LIP treatment resulted in 14% and 16% reduction ($P < 0.05$) in DMI compared to CON and GLU respectively, with no differences ($P > 0.10$) between CON and GLU treatments. The yields of milk and milk components were similar ($P > 0.10$) among treatments at both stages of lactation, however, there was a significant treatment x stage interaction ($P < 0.05$) for milk fat percentage. Relative to CON, the percentage of milk fat was reduced with GLU and increased with LIP in early lactation ($P < 0.05$) but there were no treatment differences ($P > 0.10$) in late lactation.

5.3.2. Plasma metabolites.

The overall mean concentrations and AUC for metabolites and hormones are reported in Tables 5.3 and 5.4, respectively. Temporal pattern for each hormone and metabolite is depicted in Figures 5.1 to 5.5. As expected GLU resulted in an increase ($P < 0.001$) in AUC for glucose (Table 5.4), and a sharp rise in plasma concentrations of glucose (Figure 5.1) at both stages of lactation. There was a highly significant treatment x time x stage interaction for glucose ($P < 0.001$; Table 5.3); with GLU treatment the

increase in plasma glucose concentrations over CON levels was over 4-fold by 3 h and 5-fold by 4 h in early and late lactation cows, respectively (Figure 5.1). This hyperglycemia was maintained for 7 and 8 h in early and late lactation cows, respectively, leading to a nearly 37% increase in AUC for glucose in late compared to early lactation cows. Plasma glucose concentrations, and AUC for glucose, were similar ($P > 0.10$) between CON and LIP treatments at both stages of lactation (Figure 5.1; Table 5.4). Overall glucose concentrations in CON cows tended (stage effect; $P = 0.10$) to be greater in late compared to early lactation.

Unlike glucose, overall mean concentrations and AUC for plasma NEFA were nearly 50% greater in CON cows in early compared to late lactation (stage effect; $P < 0.001$). The treatment x time x stage interaction was also significant for NEFA ($P < 0.05$; Table 5.3). Compared to CON levels, plasma NEFA concentrations were increased ($P < 0.001$) by over 4-fold within 2 h and 8-fold by 3 h of infusion of Intralipid to early and late lactation cows, respectively (Figure 5.2). As a result, relative to CON values, the AUC for NEFA were increased over 3-fold in early and late lactation cows ($P < 0.001$; Table 5.4). Although plasma NEFA concentrations did not differ ($P > 0.10$) between CON and GLU treatments at both stages of lactation, the AUC for NEFA tended ($P = 0.09$) to be reduced by 30% with GLU treatment of early lactation cows (Table 5.4). There was a treatment x time x stage interaction for plasma BHBA concentrations ($P < 0.001$ Table 5.3). With LIP treatment, plasma BHBA concentrations were increased within 3 h in both early and late lactation cows, remained elevated for at least 12 h in early lactation cows ($P < 0.05$) but did not differ ($P > 0.10$) from CON levels at 6 and 12 h in late lactation animals (Figure 5.3). In response to LIP treatment the AUC for BHBA was nearly 50% greater in early compared to late lactation cows (treatment x stage interaction, $P = 0.001$, Table 5.4). Plasma BHBA concentrations were unaffected ($P > 0.10$) by GLU treatment at both stages of lactation.

5.3.3. Plasma hormones.

Plasma concentrations of insulin were nearly 36% greater in late compared to early lactation CON cows (stage effect; $P < 0.001$). The treatment x time x stage

interaction was highly significant for insulin ($P < 0.001$; Table 5.3). In early lactation, plasma insulin concentrations were increased 3-fold over CON levels by 4 h of GLU treatment ($P < 0.001$) and remained elevated for the duration of infusion (Figure 5.4). The insulin response to GLU treatment was quite rapid and strong in late lactation animals; the concentrations increased 5-fold over CON levels within 1 h reaching a maximum 28-fold increase by 6 h of infusion ($P < 0.001$; Figure 5.4). The hyperinsulinemia with GLU was maintained for 8 and 10 h in early and late lactation cows, respectively. Compared to CON values, with GLU treatment the AUC for insulin tended ($P = 0.08$) to be increased by 22% in early lactation cows and was increased significantly ($P < 0.001$) by 450% in late lactation animals (Table 5.4). There were no differences between CON and LIP in plasma insulin concentrations at both stages of lactation ($P > 0.10$).

Plasma concentrations of leptin and IGF-1 of CON cows were 16% and 55% greater in late compared to early lactation cows, respectively (stage effect; $P < 0.001$; Table 5.3). There was a significant ($P < 0.05$) treatment x time x stage interaction for both leptin and IGF-1 (Table 5.3). Leptin and IGF-1 concentrations in plasma, and AUC for both hormones, were unaffected ($P > 0.10$) by treatment infusions in early lactation. In late lactation cows, relative to CON, plasma leptin concentrations were increased by 17% and 20% within 3 h and 6 h of infusions of LIP and GLU treatments, respectively ($P < 0.001$; Figure 5.5). Thereafter, the hyperleptinemia was maintained in both treatments for at least 12 h after the initiation of infusions. Compared to CON values, the AUC for leptin were 28% and 22% greater with GLU and LIP treatments respectively ($P < 0.05$; Table 5.4). Further, in comparison to early lactation animals, the leptin AUC of late lactation cows were 20%, 46% and 52% greater with CON, GLU and LIP treatments, respectively. In late lactation cows, plasma concentrations of IGF-1 were increased by 25% with GLU relative to CON at 6 h and remained higher thereafter until 12 h after the beginning of infusions ($P < 0.001$; Figure 5.6); however, IGF-1 concentrations did not differ ($P > 0.10$) between CON and LIP treatments. The AUC values were 24% greater with GLU relative to CON in late lactation; further, in comparison to early lactation animals, the IGF-1 AUC of late lactation cows were 82%, 104% and 91% greater with

CON, GLU and LIP treatments, respectively ($P < 0.05$). The AUC values for IGF-1 were similar among CON and LIP treatments ($P > 0.10$).

Concentrations of GH in plasma, and AUC for GH, were nearly 62% greater in early compared to late lactation CON cows (stage effect; $P < 0.001$). The treatment x time x stage interaction was significant for GH ($P < 0.01$; Table 5.3). With LIP treatment in early lactation, plasma GH concentrations were gradually reduced ($P = 0.01$) by 66% within 2 h and returned to CON levels by 8 h after the beginning of infusion (Figure 5.7), which resulted in a 25% reduction in AUC for GH (Table 5.4). Plasma GH concentrations were similar between GLU and CON in early lactation, and among all treatments in late lactation ($P > 0.10$).

5.4. Discussion

In this study we compared the effects of jugular infusions of Dextrose® (GLU) and Intralipid® (LIP) to early and late-lactation cows on DMI, endocrine and metabolic parameters, and milk production and composition. As expected, compared to cows in late-lactation, early lactation cows had greater DMI, milk yield, yields of milk components, but lower BW and BCS. Under our experimental conditions we found that short-term energy supplementation has a differential effect on DMI in dairy cows depending on the stage of lactation. Whereas GLU had no effect on DMI regardless of the stage of lactation, the hypophagic effects of LIP was specific to cows in late-lactation. Bareille and Faverdin (1996) reported that jugular infusion of lipid to early lactation cows (60 DIM), similar to the amount infused in our study, caused a reduction in DMI during the period of infusion but that the effect did not persist throughout the day. Therefore, it is possible that, in our early lactation cows, lipid infusion caused a transient reduction in DMI for the duration of infusion but the animals might have compensated post-infusion so that the daily DMI was unaffected. The specific hypophagic effect of LIP in late lactation cows concurs with the results of Bareille and Faverdin (1996) showing that lipid infusion to cows at 92 DIM causes a persistent reduction in DMI. The hormonal and metabolic mediators of this satiety effect remain to be identified.

The hyperglycemia induced by GLU treatment relative to CON levels, as

evidenced by the greater AUC's for glucose, was greater and prolonged in late compared to early lactation cows. This is suggestive of an enhanced clearance of plasma glucose in early relative to late-lactation cows, which concurs with other reports (Rose et al., 1996; Mashek et al., 2001). The increase in plasma NEFA with LIP, relative to CON, at both stages of lactation is in agreement with the report of Bareille and Faverdin (1996). Intralipid particles have similar size as chylomicrons and are hydrolysed by peripheral tissues to a similar extent as chylomicrons, resulting in an increase in plasma NEFA (Samra et al., 1998). A similar mechanism could be operating in cows used in this study because both the early and late lactation animals exhibited a massive NEFA response to LIP treatment. However, the higher basal NEFA levels coupled with a greater response to LIP treatment in early lactation animals might have overwhelmed the capacity of the liver to completely oxidize the fatty acids leading to a sharp increase in plasma BHBA concentrations. The lower magnitude of AUC for NEFA in early lactation cows receiving the GLU treatment is indicative of potential antilipolytic effects of glucose in this study. The sharp increase in plasma NEFA and BHBA with LIP treatment, in the absence of any changes in daily DMI, suggest that these metabolites do not have a persistent hypophagic effect in early lactation cows; however, the transient effects of these metabolites on DMI during the period of infusion cannot be discounted.

Compared to cows in early lactation, the insulin concentrations of CON cows were greater and GLU induced a massive insulin response in late-lactation animals similar to other reports in dairy cattle (Sartin et al., 1985; Sano et al., 1993; Gabai et al., 2002). In addition to an attenuated insulinotropic effect of glucose, insulin-mediated glucose uptake is also reported to be reduced in non-mammary tissues (adipose and muscle) of cows in early lactation thereby facilitating an increased utilization of glucose by the mammary gland (Bell and Bauman, 1997). In late-lactation cows, however, insulin-dependent utilization of glucose in adipose tissues is believed to be increased (Rose et al., 1996). Therefore, it is plausible that our early lactation cows were insensitive to insulin despite a marginal increase in plasma insulin concentrations. On the other hand, the dramatic increase in plasma insulin concentrations coupled with an enhanced tissue sensitivity might have synergized the metabolic effects of insulin in late

lactation animals. Despite marked elevation in plasma NEFA concentrations, LIP treatment had no influence on plasma insulin concentrations in this study.

It is well documented that a chronic hyperinsulinemic–euglycemic clamp results in an increase in plasma IGF-1 concentrations in early (Butler et al., 2003; Mashek et al., 2001) and mid lactation Holstein cows (McGuire et al., 1995). Further, a significant increase in IGF-1 was detectable only after 12 h of initiation of the clamp (Butler et al., 2003) whereas infusion of glucose alone had no affect on plasma IGF-1 concentrations in early lactation cows (Leonard and Block, 1997). The lack of an effect of hyperglycemia on plasma IGF-1 in our early-lactation animals is likely a consequence of the relatively short-duration of hyperinsulinemia produced by GLU infusion and (or) due to the antagonistic effects of the elevated plasma GH on insulin action. On the other hand, the increase in plasma IGF-1 concentrations with GLU treatment in late-lactation animals could be a combination of the hyperinsulinemic response, lower basal GH concentrations in plasma, and removal of the inhibitory influence of GH on insulin action. Despite a numerical increase, LIP infusion had no significant effect on plasma IGF-1 in late-lactation animals. As expected, plasma GH concentrations were greater in early compared to late- lactation cows. We provide here the first evidence of a direct inhibitory effect of LIP on plasma GH concentrations in early lactation cows, which is consistent with a similar report in sheep (Briard et al., 1998). It is likely that the low plasma GH concentrations observed in late lactation cows might have masked any inhibitory of LIP on plasma GH levels.

A novel finding of this study is the differential leptin response to short-term energy supplementation in early and late lactation cows. In support of our hypothesis, cows in late lactation were in good body condition, had greater basal leptin concentrations and exhibited a robust leptin response to short-term infusions of both GLU and LIP whereas early lactation cows had relatively lower basal leptin concentrations which were unresponsive to energy supplementation. The significant increase in plasma leptin concentrations by 6 h of infusion of GLU is consistent with studies in rodents (Koopmans et al., 1998; Levy et al., 2000a) and humans (Havel et al., 1999; Saad et al, 1998; Utriainen et al., 1996) demonstrating that 4-6 h of glucose or insulin infusions are

required to stimulate leptin secretion. The regulation of leptin production occurs at the level of transcription (Iritani, 2000), translation (Roh et al., 2003), and (or) at the level of secretion (Fain and Bahouth, 2000). If glucose directly stimulates leptin secretion then an earlier rise in circulating leptin might be expected. That the rise in insulin preceded an increase in plasma leptin would suggest that insulin mediated glucose metabolism in adipocytes coordinated the increase in leptin secretion. In support of our results, studies have shown that insulin stimulates leptin expression in bovine adipose tissue explants (Houseknecht et al., 2000), and insulin induced glucose metabolism, rather than insulin *per se*, stimulates leptin secretion in rodents (Mueller et al., 1998) and humans (Wellhoener et al., 2000). The glucose flux through the hexosamine biosynthetic pathway (Wang et al., 1998) and (or) the increased intracellular energy from glucose metabolism (Levy et al., 2000b) may be involved in the acute stimulation of leptin secretion. However, in contrast to our results, Gabai et al. (2002) observed that plasma leptin concentrations were unresponsive to jugular glucose infusions in late pregnant cows. The discrepancy between studies could be attributable to the total amount of glucose infused (723 vs 1200 gm) and the specificity of the assay system used to measure circulating concentrations of leptin (Multispecies leptin RIA kit vs bovine specific leptin RIA). The lack of an effect of GLU on plasma leptin concentrations in early lactation cows might be due to a lower hyperglycemic and hyperinsulinemic response, reduced adipose tissue sensitivity to insulin (Bell and Bauman, 1997), low BCS, and (or) a strong inhibitory influence of GH on leptin expression (Houseknecht et al., 2000).

Compared to the delayed temporal response of leptin to GLU, the leptin response to LIP treatment was more rapid in late lactation animals which is suggestive of potential differences in the mechanism of action of the two macronutrients on leptin secretion. The relatively acute increase in plasma leptin concentrations in late-lactation animals occurred in the absence of any significant changes in plasma glucose and insulin concentrations, and may play a role in mediating the satiety observed in these animals. Therefore, it is unlikely that hyperglycemia or hyperinsulinemia mediated the stimulatory effect of LIP on leptin secretion. It has been shown recently that the bovine leptin gene promoter contains functional binding sites for the transcription factors CCAAT/enhancer-

binding protein (C/EBP) and Sp1 (Taniguchi et al., 2002); and that increased activity of the hexosamine pathway in bovine endothelial cells increases Sp1 activity (Du et al., 2000). From these observations we speculate that the LIP-induced increase in leptin secretion in cattle might be through an increased activity of the hexosamine pathway. In support of this, infusion of a lipid emulsion to rats was shown to increase UDP-*N*-acetylglucosamine (UDP-GlcNAc), the end product of the hexosamine pathway, and also the gene expression and release of leptin in both muscle and fat (Wang et al., 1998). Further, basal content of UDP-GlcNAc was found to be lower in adipocytes of lean compared to obese individuals, and UDP-GlcNAc was found to induce a 21 % and 74% increase in leptin production from adipocytes of lean and obese humans, respectively (Considine et al., 2000). Although it remains to be proven, it is likely that a similar mechanism occurs in cows with the basal and lipid stimulated increase in UDP-GlcNAc, and hence of leptin production, being greater in late compared to early lactation cows.

Based on the observations in this study, and from other recent reports, we propose a mechanism through which glucose and lipid stimulate leptin secretion in dairy cattle (Figure 5.8). The stimulatory effects of glucose on leptin secretion could be direct or indirect through insulin-induced glucose metabolism in adipocytes. The increase in plasma IGF-1 concentrations might be caused either by a direct effect of glucose or mediated by hyperinsulinemia. Given the strong correlation between leptin and IGF-1 expression in adipocytes (Houseknecht et al., 2000), and potential role for IGF-1 in regulating leptin secretion (Chilliard et al., 2001), IGF-1 could directly stimulate leptin release from adipocytes or might have a permissive role in leptin secretion by attenuating the inhibitory effect of GH on leptin. Unlike the effects of glucose, the stimulatory effects of lipid on leptin secretion are direct, independent of insulin, and might be mediated through the hexosamine pathway via transcription factors such as C/EBP and Sp1. While this scenario might hold true in late-lactation cows, in early lactation animals a strong inhibitory effect of GH on leptin expression and release, in concert with low adipose reserves, might override any stimulatory effect of glucose or lipid on leptin secretion.

In summary, this study has demonstrated for the first time that plasma leptin

response to isoenergetic glucose or lipid supplementation is dependent on the stage of lactation. Holstein cows in late lactation exhibited a robust leptin response to parenteral glucose or lipid administration whereas early lactation cows were unresponsive. Both macronutrients, however, had a differential effect on leptin secretion. In comparison to the direct rapid effects of lipid infusion on leptin secretion in late-lactation animals, the glucose-induced increase in plasma leptin concentrations was delayed and was preceded by a strong insulin response. We speculate that insulin-mediated glucose metabolism may be involved in the stimulatory effects of glucose on leptin secretion but that the stimulatory effects of lipid are independent of insulin and might be mediated through the hexosamine pathway. Future studies using hyperinsulinemic and hyperglycemic clamps should be useful in delineating the functional importance of glucose metabolism in leptin regulation in ruminants. Studies are also needed to unravel the molecular events involved in the regulation of leptin secretion by lipid and glucose in ruminants.

Table 5.1. Ingredient and chemical composition of basal TMR on DM basis.

Item	
Ingredient (% DM)	
Alfalfa silage	15.97
Barley silage	19.93
Alfalfa hay	9.66
Barley grain	26.69
Ground corn	7.46
Canola meal	9.36
Soybean meal	3.77
Fishmeal	1.57
Corn gluten meal	1.36
Animal fat	1.71
Molasses	0.63
Mineral-Vitamin mix[1]	1.27
Chemical composition	Mean ± SE
DM (%)	49.93 ± 3.34
OM (%)	96.62 ± 0.47
CP (% DM)	18.13 ± 0.53
Crude fat (% DM)	3.77 ± 0.26
NDF (% DM)	30.03 ± 3.75
ADF (% DM)	17.93 ± 1.72
Ash (% DM)	0.76 ± 0.17
[2]NE_L (Mcal/kg DM)	1.70

[1]Contained (/kg DM) 125 g Na, 97 g Cl, 103 g Ca, 80 g P, 86 g Mg, 80 mg Co, 400 mg Cu, 200 mg I, 6000 mg Mn, 400 mg Se, 800 mg Zn, 3,846 KIU Vitamin A, 2,292 KIU Vitamin D, and 6,539 IU Vitamin E (Champion Feed Services Ltd., Edmonton, Canada); [2]Calculated from table values according to NRC (1989)

Table 5.2. Effect of intrajugular infusions of saline (CON), 50% dextrose (GLU) or 20% Intralipid (LIP) on dry matter intake (DMI), body weight (BW), energy balance (EB) body conditions score (BCS) and, milk composition in early and late lactation Holstein cows.

	Early Lactation				Late-lactation				*P*-value[1]		
	CON	GLU	LIP	SE	CON	GLU	LIP	SE	T	S	T x S
DMI (kg/d)	20.41	19.22	20.58	0.75	14.25	13.92	11.98	0.72	0.38	<0.001	0.06
BW (kg)	646.94	623.78	637.43	16.73	701.86	680.78	684.15	16.73	0.55	<0.001	0.92
EB (Mcal/d)	-6.55	-3.66	-4.17	1.74	2.98	4.71	5.20	1.67	0.23	<0.001	0.89
BCS	2.87	2.66	2.65	0.13	3.43	3.29	3.32	0.13	0.85	<0.001	0.83
Yield (kg/d)											
Milk	46.45	49.09	46.99	3.60	23.74	25.01	22.66	3.42	0.94	<0.001	0.96
Fat	1.88	1.63	3.18	0.24	0.92	0.90	0.94	0.22	0.13	<0.001	0.13
Protein	1.47	1.40	1.40	0.11	0.84	0.83	0.76	0.10	0.71	<0.001	0.79
Lactose	2.11	2.36	2.13	0.18	1.05	1.11	0.99	0.17	0.88	<0.001	0.88
Composition (%)											
Fat	4.05	3.29	6.72	0.47	3.83	3.59	4.06	0.42	0.19	0.03	0.04
Protein	3.19	2.88	2.97	0.12	3.58	3.29	3.32	0.10	0.07	<0.001	0.94
Lactose	4.68	4.82	4.54	0.09	4.42	4.43	4.31	0.08	0.33	0.01	0.67

[1]*P*- value for treatment and phase effects: T = treatment infusate, S = stage of lactation

Table 5.3. Effect of intrajugular infusions of either saline (CON), 50% dextrose (GLU) or 20% Intralipid (LIP) on plasma concentrations (LSM, SE) of glucose, nonesterified fatty acids (NEFA), β-hydroxybutyrate (BHBA), leptin, insulin, insulin-like growth factor-1 (IGF-1), and growth hormone (GH) in early and late lactation Holstein cows.

	Early Lactation				Late-Lactation				*P*- value[1]				
	CON	GLU	LIP	SE	CON	GLU	LIP	SE	T	S	T x S	T x H	T x H x S
Glucose (mg/dl)	59.32	162.01	64.07	13.44	70.28	207.17	80.01	8.19	<0.001	0.10	0.58	<0.001	<0.001
NEFA (μM)	329.35	227.03	904.47	46.20	173.93	177.71	763.23	48.77	<0.001	<0.001	0.28	<0.001	0.03
BHBA (mg/dl)	7.25	6.45	21.42	0.20	9.00	6.96	13.02	1.25	<0.001	0.10	0.05	0.009	<0.001
Leptin (ng/ml)	4.39	4.35	4.04	0.11	5.01	5.84	5.61	0.09	0.32	<0.001	0.10	0.11	0.008
Insulin (ng/ml)	0.21	0.38	0.22	0.05	0.29	2.18	0.27	0.09	<0.001	<0.001	<0.001	<0.001	<0.001
GH (ng/ml)	4.97	5.61	3.02	0.81	1.79	1.19	1.31	0.81	0.26	<0.001	0.27	0.005	0.006

IGF-1 (ng/ml)	68.37	71.91	68.01	8.93	106.16	131.16	114.19	8.92	0.78	<0.001	<0.001	0.21	0.04

[1]*P*- value for treatment, phase and time effects: T = treatment infusate, S = stage of lactation, H = time in hours. The covariance structure of the repeated measures was modeled as compound symmetry for leptin, IGF-1, GH and NEFA; heterogenous compound symmetry for insulin and BHBA; and first-order antedependence for glucose.

Table 5.4. Area under the curve (AUC) for profiles of hormones and metabolites of Holstein cows receiving jugular infusions of either saline (CON), 50% dextrose (GLU) or 20% Intralipid (LIP) in early and late lactation.

	Early Lactation			Late Lactation				*P*-value[1]		
	CON	GLU	LIP	CON	GLU	LIP	SE	T	S	T x S
Glucose (mmol· L^{-1}· h)	40.58	103.55	41.44	44.64	142.33	55.02	10.22	<0.001	0.03	0.19
NEFA (μmol· L^{-1}· h)	3828.31	2632.70	10598.00	1991.33	2185.87	7600.81	49.89	<0.001	<0.001	0.01
BHBA (mmol· L^{-1}· h)	8.17	8.41	26.77	7.94	8.30	15.33	1.90	<0.001	0.008	0.001
Leptin (nmol· L^{-1}· h)	6.43	6.77	6.26	7.73	9.90	9.45	0.60	0.13	<0.001	0.02
Insulin (pmol· L^{-1}· h)	747.60	912.82	645.63	975.04	5375.66	1253.90	60.59	0.01	<0.001	0.01
IGF-1 (nmol· L^{-1}· h)	151.25	166.84	157.17	275.08	341.02	300.21	45.26	0.66	<0.001	0.05
GH (μg· L^{-1}· h)	115.24	127.87	85.82	43.68	46.20	39.03	18.52	0.49	<0.001	0.05

[1]*P*- value for treatment and phase effects: T = treatment infusate, S = stage of lactation

Figure 5.1. Plasma concentrations of glucose in Holstein cows infused intrajugularly for 6 h (solid bar) with either saline (CON), 50% dextrose (GLU) or 20% Intralipid (LIP) in early (A) and late (B) lactation. Treatments differences each time point are indicated by different letters (a, b P <0.05).

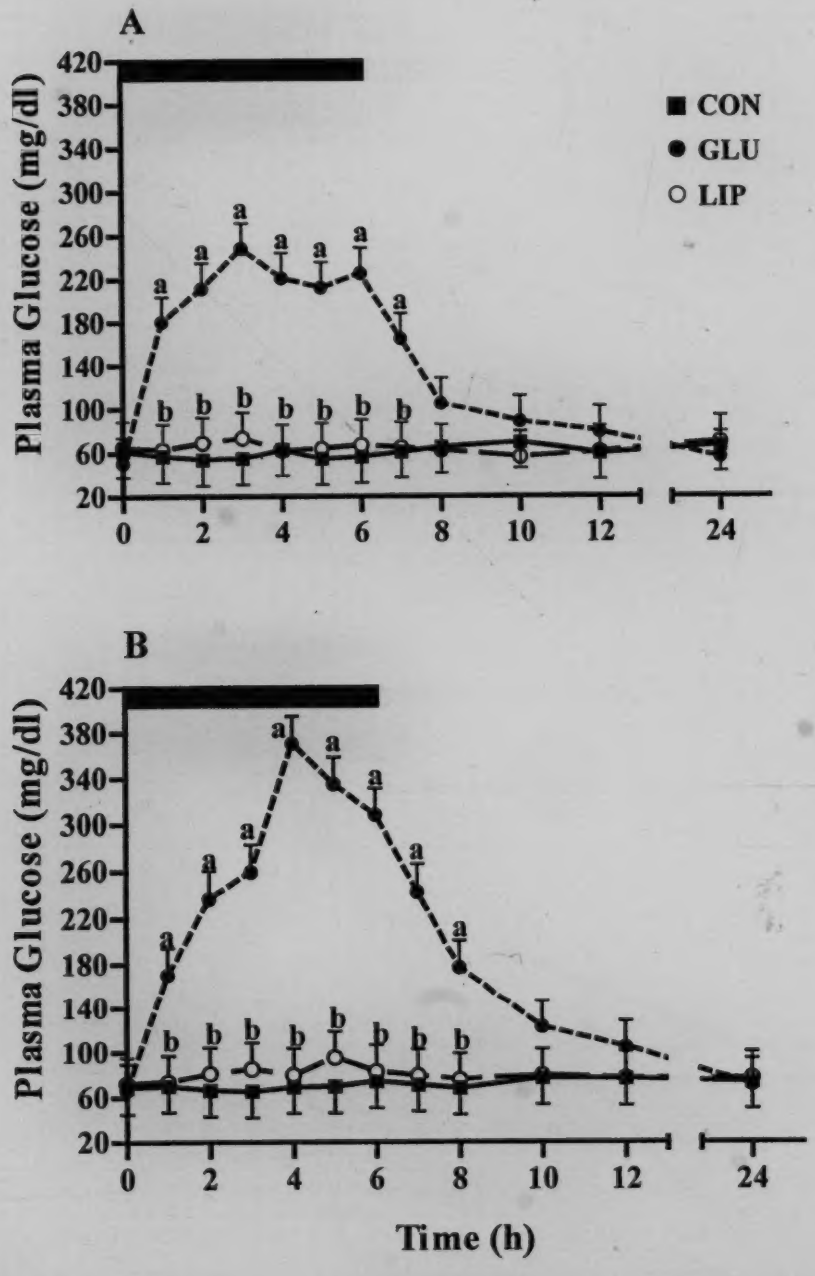


Figure 5.2. Plasma concentrations of nonesterified fatty acids (NEFA) in Holstein cows infused intrajugularly for 6 h (solid bar) with either saline (CON), 50% dextrose (GLU) or 20% Intralipid (LIP) in early (A) and late (B) lactation. Treatments differences each time point are indicated by different letters (a, b $P < 0.05$).

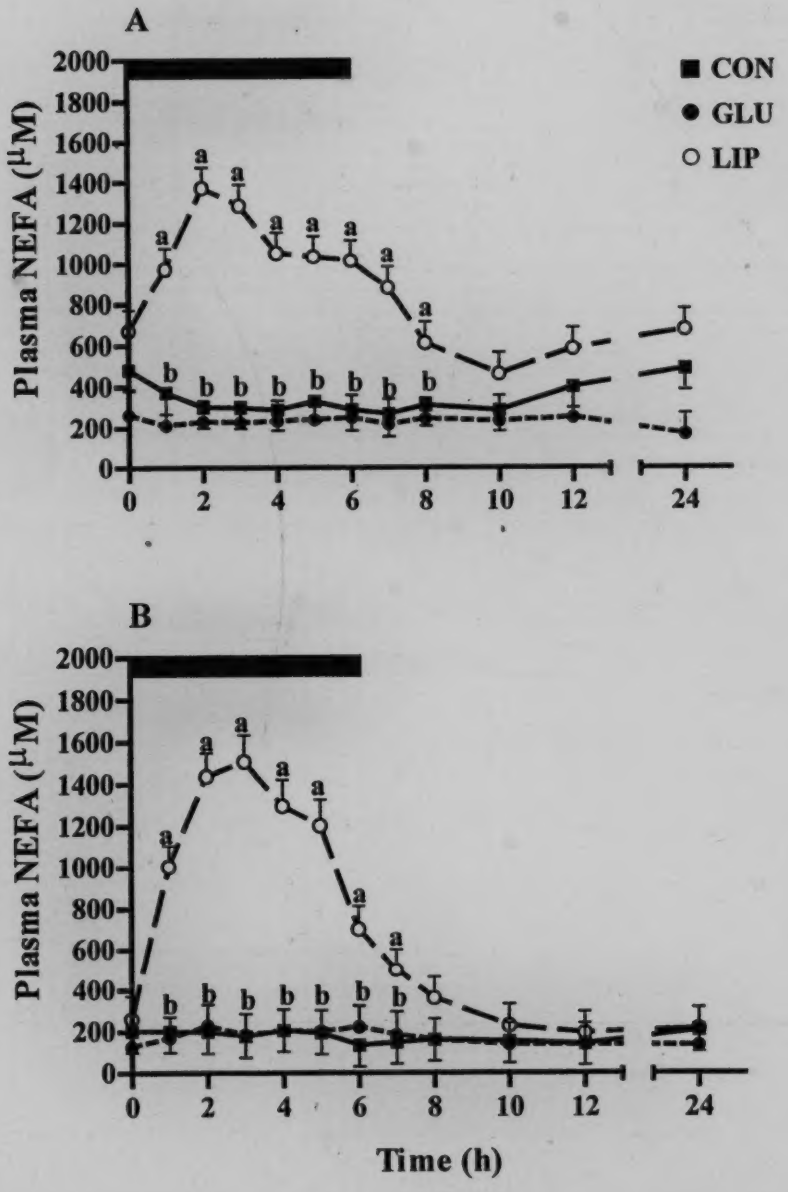


Figure 5.3. Plasma concentrations of β-hydroxybutyrate (BHBA) in Holstein cows infused intrajugularly for 6 h (solid bar) with either saline (CON), 50% dextrose (GLU) or 20% Intralipid (LIP) in early (A) and late (B) lactation. Treatments differences each time point are indicated by different letters (a, b P <0.05).

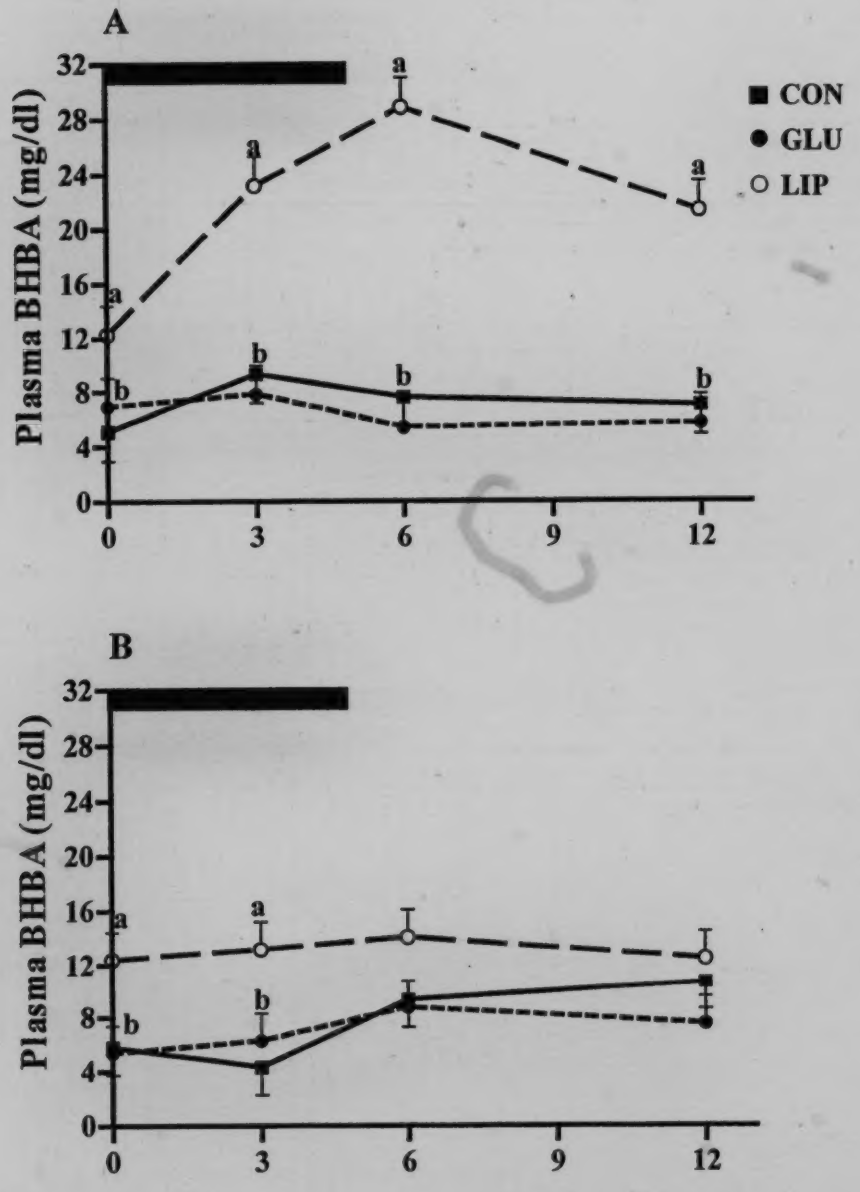


Figure 5.4. Plasma concentrations of insulin in Holstein cows infused intrajugularly for 6 h (solid bar) with either saline (CON), 50% dextrose (GLU) or 20% Intralipid (LIP) in early (A) and late (B) lactation. Treatments differences each time point are indicated by different letters (a, b $P < 0.05$).

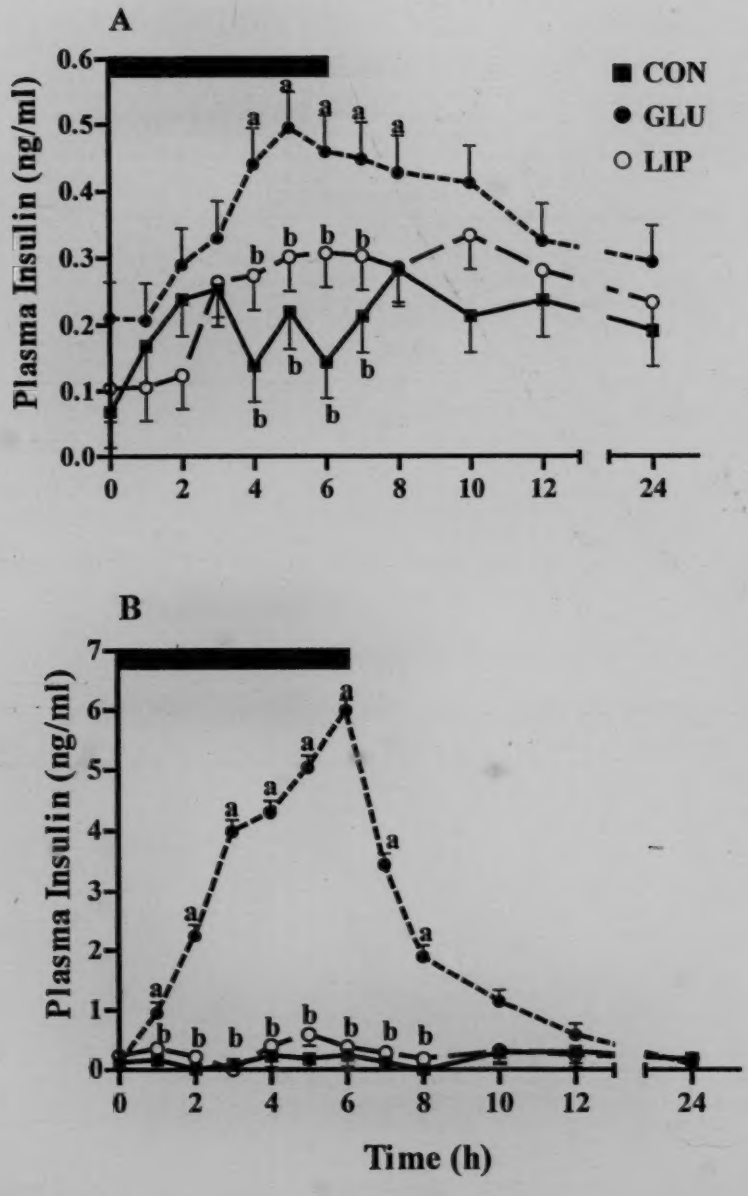


Figure 5.5. Plasma concentrations of leptin in Holstein cows infused intrajugularly for 6 h (solid bar) with either saline (CON), 50% dextrose (GLU) or 20% Intralipid (LIP) in early (A) and late (B) lactation. Treatments differences each time point are indicated by different letters (a, b P <0.05).

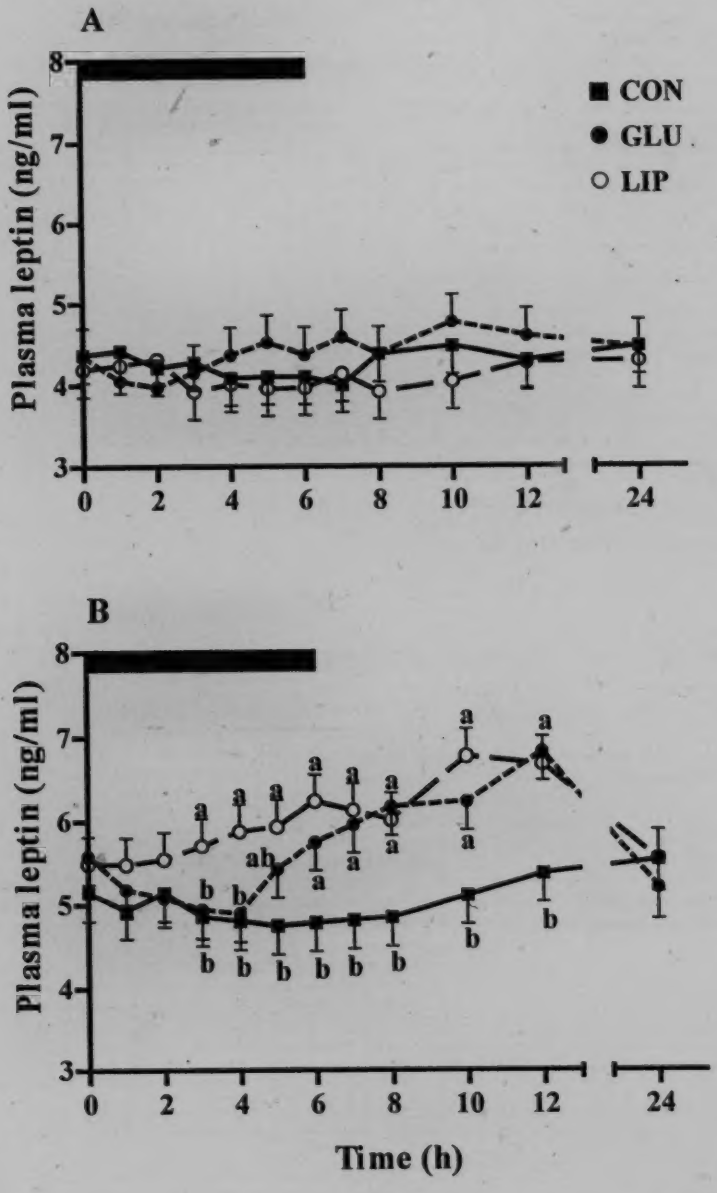


Figure 5.6. Plasma concentrations of insulin-like growth factor-1 (IGF-1) in Holstein cows infused intrajugularly for 6 h (solid bar) with either saline (CON), 50% dextrose (GLU) or 20% Intralipid (LIP) in early (A) and late (B) lactation. Treatments differences each time point are indicated by different letters (a, b P <0.05).

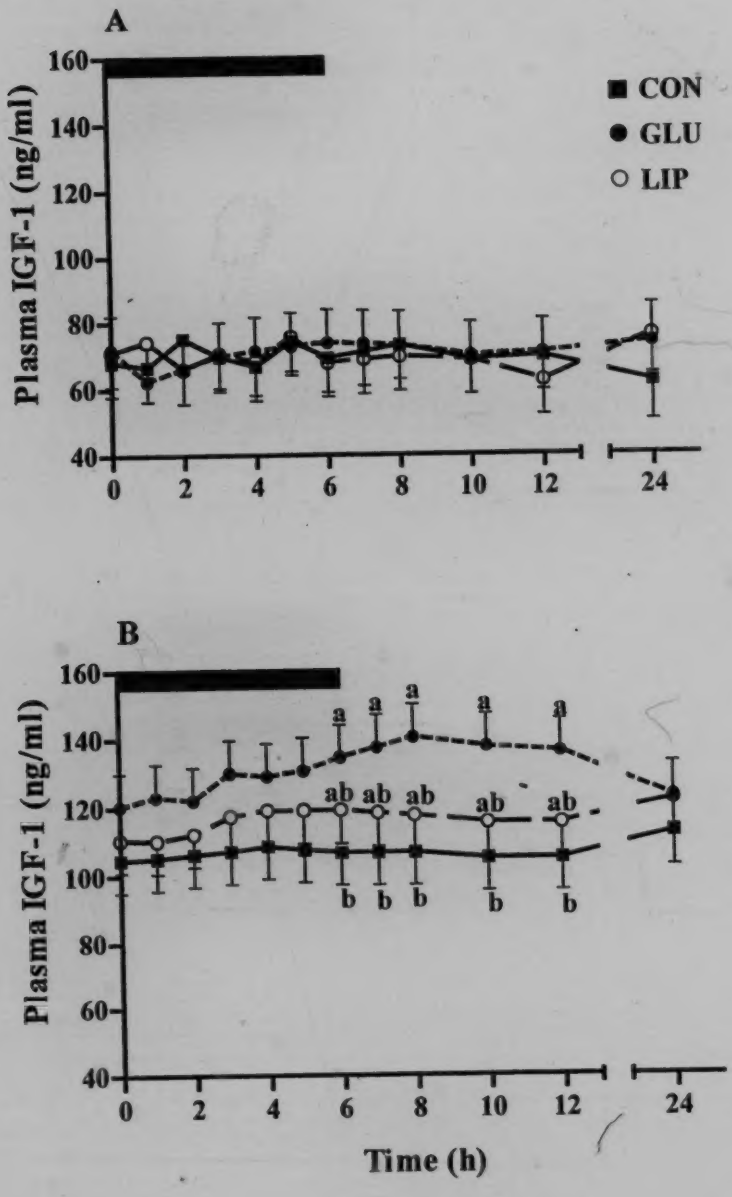


Figure 5.7. Plasma concentrations of growth hormone (GH) in Holstein cows infused intrajugularly for 6 h (solid bar) with either saline (CON), 50% dextrose (GLU) or 20% Intralipid (LIP) in early (A) and late (B) lactation. Treatments differences each time point are indicated by different letters (a, b $P < 0.05$).

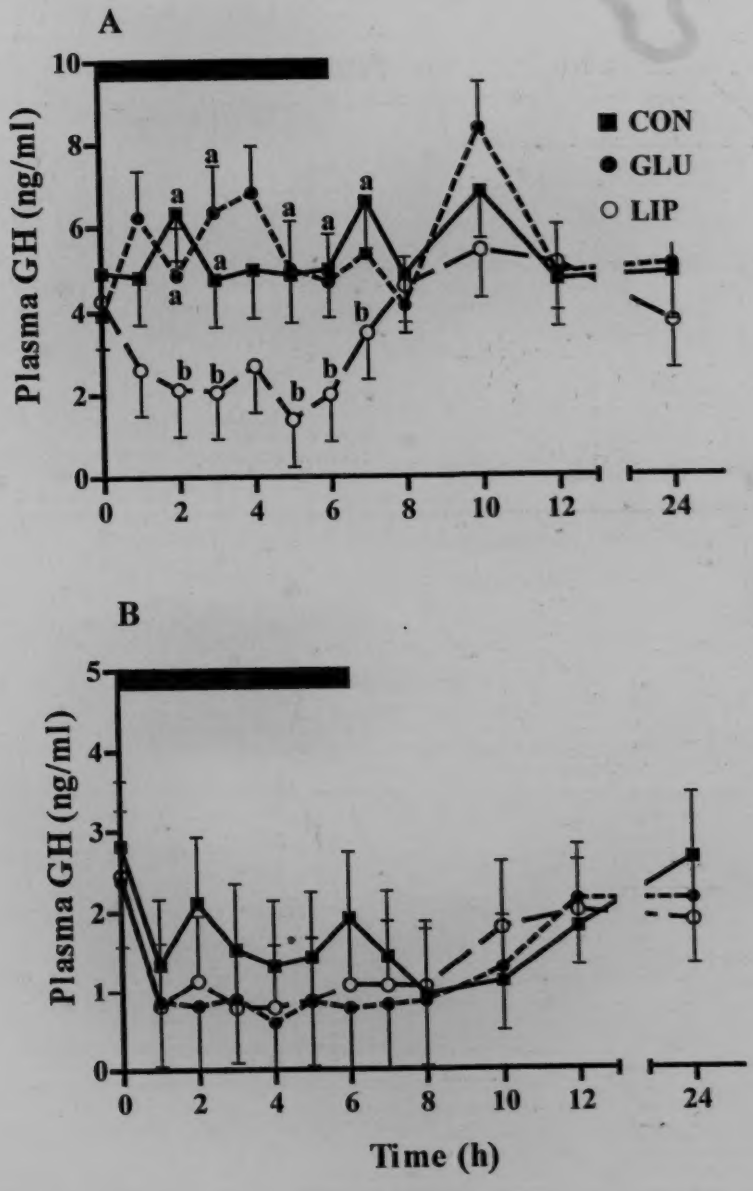
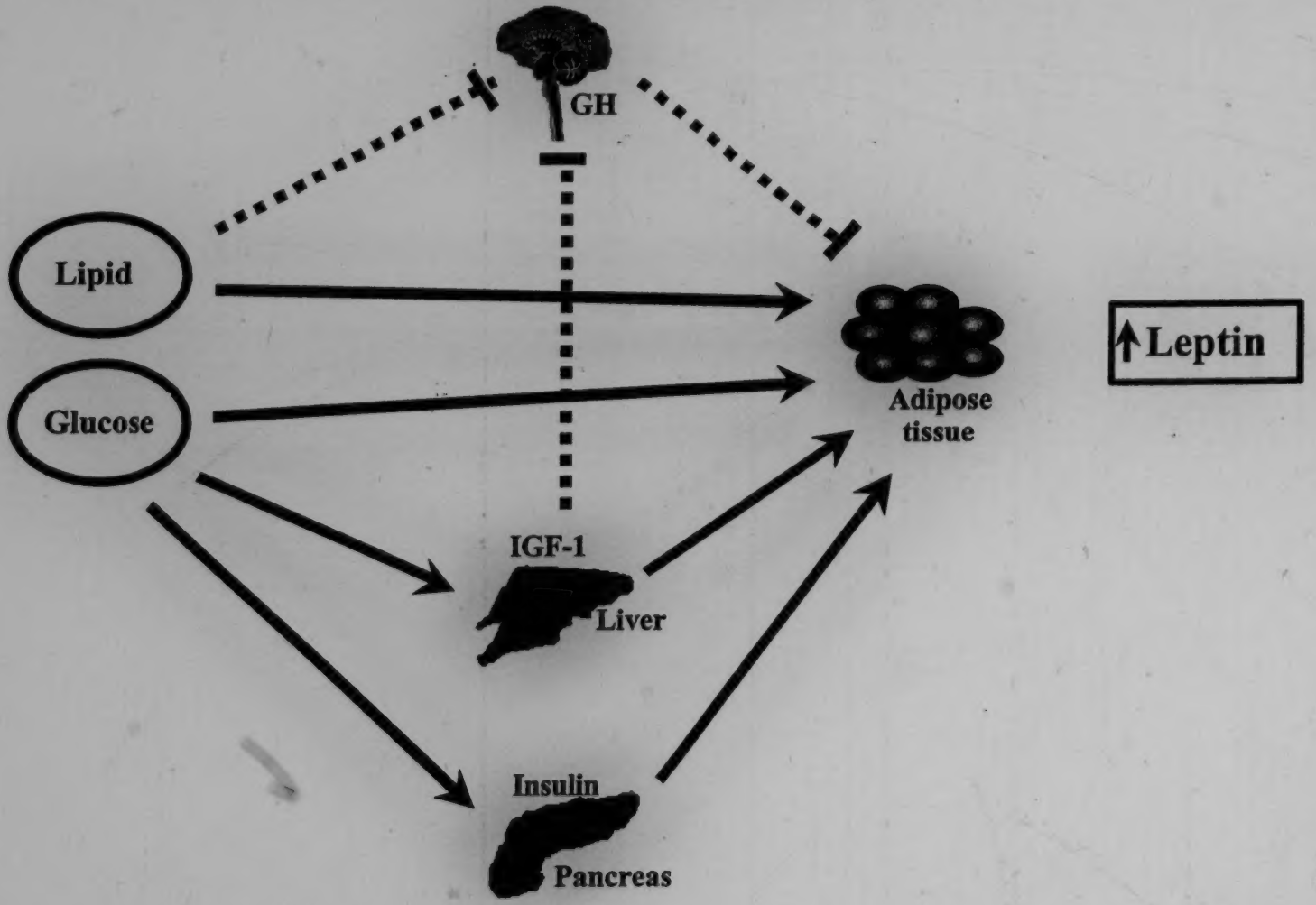


Figure 5.8. Proposed mechanisms for the effects of glucose and lipid on leptin secretion in cattle. Glucose could stimulate leptin secretion directly or indirectly through an increase in circulating concentrations of insulin and IGF-1. In addition to a direct stimulatory effect, IGF-1 might play a permissive role by attenuating the inhibitory effect of GH on leptin secretion. The stimulatory effects of lipid on leptin secretion are direct and independent of insulin or IGF-1. Whereas this scenario may hold true in late lactation animals; in early-lactation cows a strong inhibitory effect of GH on leptin expression and release, in concert with low adipose reserves, might override any stimulatory effect of glucose or lipid on leptin secretion. Stimulatory effects are depicted by arrows and inhibitory effects by broken lines.


GH
Lipid
Glucose
Adipose tissue
Leptin
IGF-1
Liver
Insulin
Pancreas

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CHAPTER 6

Effect of dietary energy and protein density on body composition, attainment of puberty, ovarian follicular dynamics and, peripubertal changes in plasma concentrations of leptin and other hormones and metabolites in dairy heifers[1]

6.1. Introduction

Rapid prepubertal growth rates hasten the onset of puberty but have a detrimental effect on mammary development and milk production potential of dairy heifers (Sejrsen et al., 2000). There is substantial evidence linking the allometric growth of the mammary gland to maturation of the reproductive tract during the prepubertal period (Lammers et al., 1999; Maple et al., 1998; Purup et al., 1993). Despite such evidence relating mammary development to reproductive maturity, and the effects of nutrition on mammary development, there are no studies documenting the effects of diet on ovarian follicular dynamics in dairy heifers during the pre or peripubertal period. Further, the signals that communicate the reserves of body fat to the brain to initiate puberty in dairy heifers are not completely understood.

To date, research on the normal physiological changes in ovarian follicular dynamics (Melvin et al., 1999; Adams et al., 1994; Evans et al., 1994) or the effects of nutritional manipulation on follicular dynamics, hormonal and metabolite profiles during the peripubertal period (Bergfeld et al., 1994) or postpuberty (Armstrong et al., 2001; Bossis et al., 2000; Bossis et al., 1999; Mackey et al., 1999; Rhodes et al., 1995) has been confined to studies on beef heifers. In these animals, a low plane of nutrition inhibits LH secretion, delays development of dominant follicles and puberty (Kinder et al., 1995), and alters body composition at puberty (Simpson et al., 1998; Hall et al., 1995; Yelich et al., 1995). Among the hormonal signals, a prepubertal increase in circulating concentrations of insulin-like growth factor-1 (IGF-1) (Yelich et al., 1995) and leptin (Garcia et al., 2002, 2003) are believed to play a role determining the onset of puberty in beef heifers.

[1] A version of this chapter has been published in Theriogenology 2003; 60: 707-725

The objectives were to determine the effects of diets differing in energy and protein density on age, growth parameters (body weight, back-fat thickness, and body condition score), body composition at puberty, temporal changes in plasma concentrations of leptin, insulin, IGF-1, growth hormone (GH), glucose, nonesterified fatty acids (NEFA), and LH characteristics and ovarian follicular dynamics during pre and peripubertal periods. We also assessed the effects of realimentation of feed-restricted prepubertal heifers on pubertal parameters such as age, weight, growth, body composition and ovarian follicular characteristics.

6.2 Materials and methods

6.2.1. Heifers, design and treatments

The experiment was conducted from April 1999 to October 2000 at the Dairy Research and Technology Centre, University of Alberta, with all animal procedures approved by the University of Alberta Animal Policy and Welfare Committee (protocol number 99-09 C). This study was conducted in two phases. Thirty Holstein heifer calves entered Phase 1 (P1) of the study following a 10 d adaptation period during which the calves were fed a diet containing (on a dry matter basis) 40 % alfalfa hay, 10 % barley silage and 50 % barley grain. At 103 ± 2 kg body weight (BW), and 104 ± 2 d of age, the calves were assigned at random to one of three dietary treatments (n = 10/treatment). The diets (Table 6.1) were formulated using the Cornell Net Carbohydrate and Protein System (Van Amburgh et al., 1998) and limit-fed for a predicted average daily gain (ADG) of 0.50 kg/d (Low: P1L), 0.80 kg/d (Medium: P1M) or 1.10 kg/d (High: P1H). We anticipated that heifers on the medium diet would achieve a postpubertal BW of 330 kg at approximately 12 mo of age, at which stage they were allowed ad libitum access to a single Phase 2 (P2) diet to maintain their ADG of 0.8 kg/d. To study treatment differences at a similar age and weight, five heifers each within the High and Low groups were assigned to P2 at either 12 mo of age (P2H-12; P2L-12) or entered P2 when they attained 330 kg BW (P2H-330; P2L-330) (Figure 6.1). Heifers were housed in tie-stalls, exercised thrice weekly between 0900 h and 1000 h, and fed individually a total mixed ration (TMR) once a day at approximately 1000 h with free access to water.

6.2.2. Measurements, sampling and analyses

6.2.2.1. Diets

Daily feed consumption was recorded for each heifer. Based on the weekly average daily gain, the amount of feed allotted to each heifer in Phase 1 was adjusted to achieve the target growth rates, whereas phase-2 heifers were fed in excess of what they would consume. Samples of TMR, ingredients and orts were collected once weekly, dried at 55 °C for 72 h, ground through a 1 mm screen (Thomas-Wiley Laboratory Mill Model 4, Philadelphia, PA, USA) and composited monthly for proximate analysis. Dry matter (DM) was determined by drying samples at 110 °C overnight, and organic matter (OM) was calculated as weight loss upon ashing for at least 6 h at 500 °C. Samples were analyzed for crude protein (CP: 6.25 x N; Leco FP-428 nitrogen determinator, Leco® Corporation, St. Joseph, MI, USA), neutral detergent fiber (NDF), acid detergent fiber (ADF), and lignin (Ankom filter bag technique®, Ankom Company, NY, USA).

6.2.2.2. Growth measurements

The animals were weighed on two consecutive days weekly before feeding and the mean weekly body weight calculated. When animal reached 200 kg BW, and every two weeks thereafter, body condition score (BCS) and back-fat thickness (between 12^{th} and 13^{th} rib) were recorded. The BCS was assessed on a scale of 1 to 5 (Edmonson et al., 1989) by two individuals, and back-fat thickness was measured transcutaneously using an ultrasound scanner (Aloka® 500V, Aloka Co., Ltd., Tokyo, Japan) equipped with a 7.5 MHz linear array transducer (Brethour, 1992). Indirect estimates of skeletal growth were made by measuring height at withers, height at hip, heart girth, and body length from point of shoulders to pin bones using a measuring tape. These growth measurements were recorded every two weeks by the same two individuals for the entire duration of the study.

6.2.2.3. Body composition

At monthly intervals between 8 and 10 mo of age, and again at the end of each

phase of the study, body composition was estimated indirectly by the urea dilution technique (Koch and Preston, 1979). Briefly, heifers were fitted with jugular catheters (polyethylene tubing ID 1.67 mm, OD 2.42 mm, Intramedic PE 240, Clay Adams®, Becton Dickinson, Sparks, MD, USA) on the day before urea space estimation and kept patent overnight with 200 IU/ml of heparinized saline. A 20 % solution of urea in 0.9 % saline solution (wt/vol) was freshly made and sterile-filtered through 0.22 µm Millipore® filters (Molsheim, France). Following an overnight fast (18 to 20 h), to reduce gut-fill, shrunk body weights were recorded and the urea solution in preweighed sterile syringes was infused via the catheters at the rate of 0.66 ml/kg BW (i.e. 130 mg urea/kg BW) within 2 min. Duplicate blood samples were collected on ice at 0.5 min before the start of infusion (initial sample) and at 12 min from the midpoint of infusion (final sample) in 10 ml heparin tubes (Becton Dickinson). Prior to collecting blood samples, the catheters were flushed with 10 to 20 ml of 20 IU/ml heparinized saline. Within 2 h of sampling, plasma was separated by centrifugation (1500 x g) and stored at – 20 °C along with an aliquot of the stock urea solutions. Urea nitrogen in plasma (PUN) and stock solutions were analysed using Infinity™ BUN liquid reagent (63-2000P, Sigma Chemical Co., St. Louis, MO). Urea space was calculated as a percentage of shrunk BW (SBW) by the following equation (Hammond et al., 1990): Urea space % = [(concentration of stock solution) x (actual volume of solution infused)]/(Final PUN - Initial PUN) x SBW x 10). From the urea space values, percentages of empty body fat and protein were calculated using multiple regression equations (Hammond et al., 1990), and body composition at puberty was calculated using these regressions.

6.2.2.4. Ovarian ultrasonography

To characterize ovarian follicular and corpus luteum (CL) dynamics, a subset of heifers were selected at random from each of the P1H, P1M, and P1L treatments (n = 5) and examined in two observational periods at 8 and 10 mo of age. Transrectal ultrasonography was performed on alternate days for 21 d at 8 mo, and for 50 d at 10 mo of age. These periods were chosen to encompass the pre- (8 mo) and peripubertal (10 mo) periods for the high and medium treatment groups. To compare treatments at a

similar physiological stage, that is at puberty, ovaries of heifers on the low diet were scanned on alternate days over a 70 d period beginning at 12 mo of age when the heifers were allowed ad libitum access to the Phase 2 diet (P2L-12). An Aloka® 500V ultrasound scanner equipped with a 7.5 MHz linear array transducer was used for recording the position and size of follicles and CL on both ovaries. Based on diameter, the follicles were counted and classified (Lucy et al., 1992) as: class 1 (≤ 5mm), class 2 (6 to 9 mm) or class 3 (≥ 10 mm). The waves of growth of dominant and subordinate follicles on each ovary were identified from a retrospective examination of ovarian maps, and the follicular and CL dynamics were defined based on established criteria (Ireland et al., 2000). The waves were aligned to the day of emergence and consecutively numbered. The follicle and CL diameters were calculated as the mean of the largest and smallest diameters. The size of luteal tissue was calculated by subtracting the mean diameter of the luteal cavity from the mean outer diameter of the CL. The growth rate of a follicle or CL was calculated as the difference between the maximum and minimum diameter divided by the number of days of growth. The regression rate was calculated as the difference between the maximum size prior to initiation of regression and the minimum size detectable divided by number of days of regression. The lifespan of a follicle or CL was defined as the number of days the individual follicle or CL > 4 mm was detectable.

6.2.2.5. *Blood sampling and analyses*

Blood samples were collected from all heifers at 2-week intervals throughout the study. Further, beginning at 200 kg BW, blood samples were collected twice weekly (Monday and Friday) via jugular vein puncture from all heifers. Samples were collected on ice in 10 mL EDTA vacutainer tubes (Becton Dickinson), plasma separated by centrifugation at 1500 x g for 20 min and stored at – 20 °C.

Plasma progesterone concentrations in all samples were determined with a solid-phase radioimmunoassay (RIA) kit (Coat-a-Count, Intermedico, Markham, ON, Canada) using a 200 µL sample volume. For the low (2.17 ng/mL) and high (4.19 ng/ml) control

plasma, the intra-assay CV were 7.77 and 10.22 % respectively, and the inter-assay CV for 24 assays were 11.93 and 6.06 %, respectively. Assay sensitivity was 0.02 ng/ml. Serial dilutions of pooled bovine plasma produced curves parallel to standard curves. Addition of 2, 20 and 40 ng/ml of progesterone standards to a plasma pool resulted in recoveries of 96 %, 102 % and 104 % respectively. We considered that a heifer was pubertal, and first ovulation had occurred, when plasma progesterone concentrations exceeded 1 ng/mL (Honaramooz et al., 1999) for the first time. In heifers that were subjected to transrectal ultrasonography, puberty was further confirmed by the formation of a detectable CL. All plasma samples were analyzed for leptin, insulin, and GH according to the procedures described in Chapter 3. For samples collected twice weekly, the mean of both samples was considered as the weekly mean. Analyses for plasma glucose, NEFA and IGF-1 concentrations were done only in samples that were collected at fortnightly intervals.

To determine pulsatile characterisctics of leptin and LH, blood samples from six heifers from each of the P1H, P1M and P1L treatment groups were taken via jugular catheters at 15 min intervals for 8 h at 8 mo and again at 10 mo of age. Heifers were fitted with jugular catheters on the day before sampling as for urea space measurements. Blood samples were collected on ice in 10 mL EDTA vacutainer tubes (Becton Dickinson), plasma separated by centrifugation at 1500 x g for 20 min and stored at - 20° C. Plasma LH concentrations were quantified by an established RIA (Endocrine Lab Services, University of Saskatchewan, Saskatoon, SK, Canada). For the low (0.38 ng/ml) and high (1.01 ng/ml) control plasma the intra-assay CV were 4.75 and 3.57 % respectively, and the inter-assay CV from 5 assays were 4.12 and 4.83 % respectively. Assay sensitivity was 0.1 ng/mL. The Kushler-Brown PulseFit program (Kushler and Brown, 1991), a stepwise selection algorithm for identifying hormone pulses, was used to characterize leptin and LH pulsatility and obtain values for pulse frequency, amplitude, mean and basal concentrations. In the first step a set of pulses are identified using the CV of the observed data, and an estimate of pulse parameters (baseline concentration and rate of removal of excess hormone from circulation) is obtained by non-linear regression. Then a test for presence of 'superpeaks' (extremely high values) is done and the

parameter estimates revised using non-linear regression. Based on the revised parameter estimates the program adds/deletes pulses by stepwise linear regression. The procedure alternates between stepwise regression and non-linear fitting until no pulses are added or deleted. The parameters specified for the pulse detection algorithm were: 'trimming fraction' for obtaining initial error estimate = 0.20 (determined from the data), 'initial critical value' for initial identification of pulses = 2.00 (larger value causes the algorithm to start with fewer pulses and perform forward addition), 'tuning constant' for stepwise identification of pulses = 2.00 (larger value makes the stepwise search more conservative), and testing for 'super-peaks'.

6.2.2.6. Statistical analyses

Data were analyzed by ANOVA for a completely randomized design using the MIXED procedure of SAS (release 8.0). Repeated measures on plasma hormone and metabolites, follicle characteristics, growth and body composition measurements, and dominant follicle characteristics were analyzed using the following univariate linear mixed model:

$$Y_{ijk} = \mu + \alpha_i + \beta_{ij} + \gamma_k + (\alpha\gamma)_{ik} + e_{ijk}$$

where μ is the population mean, α_i is a population parameter corresponding to treatment i, β_{ij} is a random variable corresponding to heifer j in treatment i, γ_k is a parameter corresponding to day k, $(\alpha\gamma)_{ik}$ is an interaction parameter corresponding to treatment i and day k, and e_{ijk} is the residual error. Based on the lowest values of fit statistics for AIC, AICC and BIC criteria, an appropriate covariance structure was fitted for each repeated measurement (Littell et al., 2000). For profiling follicular dynamics, when $(\alpha\gamma)_{ik}$ was found to be significant ($P < 0.05$) then day was treated as a continuous variable and polynomial regression curves were used to model treatment responses as a function of day (Littell et al., 2000). Further, treatment differences at individual time points were obtained by using the Estimate statement. If $(\alpha\gamma)_{ik}$ was not significant, and for discrete variables, the treatment effects were analyzed using the Estimate statement with the Satterthwaite option. Stepwise regression procedure was used for determining the

relative contribution of variables such as ADG, BW, BCS, BF, empty body fat and protein percent, to variation in age at puberty. Comparisons with $P < 0.01$ were declared highly significant, $P < 0.05$ significant, and $0.05 < P < 0.10$ were considered as trends.

6.3. Results

6.3.1. Diets

To maintain a consistent chemical composition of the diets for the entire duration (~16 mo) of the trial, the proportion of ingredients was adjusted as and when the source of forage changed. The ingredients and overall mean chemical composition of the diets fed to heifers are presented in Table 6.1.

6.3.2. Growth

The age (P1H: 111.1± 4.7 d; P1M: 97.8 ± 5.0 d; P1L: 104.0 ±4.5 d) and body weight (P1H: 107.0 ± 4.4 kg; P1M: 104.2 ± 4.6 kg; P1L: 100.8 ± 4.2 kg) at which the heifers began the study did not differ ($P > 0.10$) among the treatment groups. The height at withers (P1H: 82.0 ± 1.5 cm; P1M: 84.8 ± 1.5 cm; P1L: 84.2 ± 1.5 cm), height at hip (P1H: 85.8 ± 2.1 cm; P1M: 88.8 ± 1.3 cm; P1L: 87.2 ± 0.8 cm), body length (P1H: 66.4 ± 2.3 cm; P1M: 68.4 ± 1.3 cm; P1L: 67.7 ± 0.8 cm) and heart girth (P1H: 108.8 ± 4.5 cm; P1M: 108.2 ± 2.5 cm; P1L: 110.0 ± 1.5 cm) at the initiation of the study were similar ($P > 0.10$) among the treatment groups. All heifers fed the high (ADG 1.04 kg/d) or medium diets (ADG 0.83 kg/d) attained puberty in Phase 1 of the trial with no differences ($P > 0.10$) between subgroups in pubertal BW, age, ADG, back-fat thickness and growth measurements. Hence, data on these parameters from subgroups were combined and are shown under P1H and P1M in Table 6.2. The ADG and age at puberty differed ($P < 0.01$) among treatments as shown in Table 6.2. Heifers fed P1H, P1M, P1L and P2L-12 diets attained puberty at approximately 9, 11, 16 and 14 mo of age, respectively ($P < 0.001$). The body weight and back-fat thickness at puberty did not differ among treatments ($P > 0.10$). The BCS at puberty differed ($P < 0.05$) among treatments with LP1 heifers having the lowest BCS followed by P2L-12 heifers ($P < 0.05$) with no

differences between P1H and P1M heifers.

6.3.3. Body composition

There was a significant ($P < 0.05$) treatment by time interaction for urea-space derived estimates of body fat and protein percentages from 8 to 12 mo of age. Heifers on the P1L diet had lower ($P < 0.05$) body fat percent compared to P1H or P1M heifers, with no differences ($P > 0.10$) between P1H and P1M heifers from 9 to 11 mo of age (Figure 6.2 -A). Treatments differed ($P < 0.05$) in the estimates of body protein, with P1H heifers having greater body protein percent (Figure 6.2-B). At 12 mo of age, P1H heifers had greater body fat and protein percent compared to P1L heifers ($P < 0.05$) with no differences ($P > 0.10$) between P1M and P1L heifers. However, heifers attained puberty at similar body fat and protein percent (Table 6.2). Indirect estimates of skeletal growth indicated that relative to P1H, P1M or P2L-12 heifers, P1L heifers were taller ($P < 0.05$) at the hip and withers. Both P1L and P2L-12 heifers had greater ($P < 0.05$) heart girth compared to P1H or P1M heifers, but all heifers had similar ($P > 0.10$) body length. Stepwise regression analysis revealed that ADG, BW, and body fat percent explained 98 % of the variation in age at puberty ($P < 0.09$). ADG and BW together contributed to 96 % of the variation ($P < 0.001$), but the inclusion of body fat percent to the model tended ($P < 0.09$) to increase r^2 to 98%. Back-fat thickness, empty body protein percent and BCS did not contribute significantly ($P > 0.10$) to the variation in age at puberty.

6.3.4. Ovarian ultrasonography

Data on ovarian follicular dynamics during the prepubertal period, at 8 months of age, are presented in Table 6.3 and Figure 6.3 (A). The mean number of class 1 ($\leq$ 5mm) and 2 (6 to 9 mm) follicles did not differ ($P > 0.10$) among treatments, but P1L heifers had fewer ($P < 0.05$) class 3 ($\geq$ 10 mm) follicles than P1H or P1M heifers (Table 6.3). The growth rate, regression rate, and life span of the dominant and subordinate follicles did not differ ($P > 0.10$) among treatments. The mean diameter of the dominant follicle was smaller in P1L heifers ($P < 0.05$) compared to P1H or P1M heifers, but the size of subordinate follicles did not differ among treatments ($P > 0.10$). Since treatment by day

interaction was significant ($P < 0.001$) for the diameter of the dominant follicle, regression curves were used to depict the time–dependent changes in dominant follicle characteristics in a wave (Figure 6.3 A). Compared to P1H or P1M heifers, the diameter of the dominant follicle was smaller in P1L heifers between Days 11 and 13 of the observational period.

Follicular characteristics during the peripubertal period in P1H, P1M and P2L-12 heifers are presented in Table 6.4 and Figure 6.3 (B). The mean number of class 1 follicles was greater in P2L-12 heifers compared to P1H or P1M heifers ($P < 0.05$). The mean number of follicles in classes 2 and 3 did not differ among treatments ($P > 0.10$). The mean maximum size and regression rate of the nonovulatory dominant and subordinate follicles, and of the penultimate wave before first ovulation, did not differ ($P > 0.10$) among treatments. However, the effect of age was significant ($P < 0.05$) with mean maximum size and growth rate of the nonovulatory dominant follicle being greater at 10 mo than at 8 mo of age in both P1H and P1M heifers. Because treatment x day interaction was highly significant ($P < 0.001$) for the diameter of the nonovulatory dominant follicle, polynomial regression curves were used to profile the follicular characteristics (Figure 6.3 B). From Day 9 to Day 15 of the penultimate nonovulatory wave, P2L-12 heifers had larger ($P < 0.05$) nonovulatory dominant follicles compared to P1H or P1M heifers. The growth rate of the nonovulatory dominant follicle was greater in P2L-12 heifers compared to P1M heifers ($P < 0.05$) but did not differ ($P > 0.10$) from P1H heifers. The subordinate follicle also grew more rapidly in P2L-12 heifers than P1H or P1M heifers ($P < 0.05$). The lifespan of the nonovulatory dominant, subordinate and ovulatory follicles were similar among treatments ($P > 0.10$). The size of the first and second ovulatory follicles was larger in P2L-12 than in P1M heifers ($P < 0.05$), but did not differ between P2L-12 and P1H heifers ($P > 0.10$). The first ovulatory follicle grew more rapidly in P2L-12 than P1H heifers ($P < 0.05$). The third ovulatory dominant follicle had similar characteristics among treatments ($P > 0.10$). When compared to P1H and P1M heifers at a similar chronological age (10 mo), P1L had less number of class 1 ($P < 0.01$) and class 3 follicles ($P < 0.01$). Further, the maximum size, growth and

regression rates of the nonovulatory dominant ($P < 0.05$) and subordinate follicles ($P < 0.05$) were also reduced in P1L heifers compared to P1H or P1M heifers.

Characteristics of the first and second ovulatory cycles are shown in Table 6.5. The lifespan, maximum size and regression rate of the first and second CL did not differ among treatments ($P > 0.10$). The first CL grew more slowly in P2L-12 heifers ($P < 0.05$) compared to P1H or P1M heifers. When compared to the second ovulatory cycle, the maximum size of the first CL was smaller, the life span of the first CL shorter, and the first interovulatory interval was of shorter duration across treatments ($P < 0.05$).

6.3.5. Plasma hormones and metabolites

Plasma LH characteristics at 8 and 10 mo of age are shown in Figure 6.4. Among the 6 heifers from each group that were frequently tested, two P1H heifers at 10 mo, and one P1M heifer at 10 mo of age, were found to have attained puberty as determined by retrospective examination of plasma progesterone concentrations and ovarian maps. Another P1H heifer had abnormal ovarian characteristics with very few class 2 and no class 3 follicles, no CL either at 8 or 10 mo, undetectable plasma progesterone concentrations, and an abnormal LH profile. Data from these animals were not considered for analysis, reducing the group size to 3 P1H and 5 P1M heifers. Despite numerical increases in mean and basal LH concentrations with age, there were no treatment differences ($P > 0.10$). At 8 mo of age, P1H and P1M heifers had greater pulse amplitude ($P < 0.05$) compared to P1L heifers but similar ($P > 0.10$) pulse frequency. Heifers on the P1H diet had high frequency low amplitude pulses at 10 mo of age compared to heifers on the P1L diet ($P < 0.05$). With age, there was a tendency ($P < 0.09$) for pulse frequency to increase and amplitude to decrease in both P1H and P1M heifers. Overall mean plasma progesterone concentrations following first ovulation were similar between P1H and P1M heifers (P1H: 2.01 ± 0.28 ng/ml; P1M: 1.55 ± 0.16 ng/ml) with no differences between subgroups. Though mean plasma progesterone concentrations did not differ ($P > 0.10$) among treatments in the first ovulatory cycle (Table 6.5), peak progesterone concentrations were lower in P2L-12 relative to P1H or P1M heifers ($P < 0.05$). Independent of treatment effects, overall mean plasma

progesterone concentrations in the first ovulatory cycle (1.68 ± 0.30 ng/ml) were lower ($P < 0.05$) than the concentrations in the second ovulatory cycle (2.58 ± 0.30 ng/ml)

The plasma leptin profile for the entire duration of the study is shown in Figure 6.5 A, and the changes during the peripubertal period are shown in Figure 6.5 B. During phase 1, there were no treatment differences between subgroups at similar time points. Compared to high group heifers, plasma leptin concentrations were reduced ($P < 0.05$) in the L group heifers within 10 weeks since the beinning of the study, and these differences followed the changes in body weight and back-fat thickness (Figure 6.7). After switching, to the phase-2 diet, plasma leptin concentrations rapidly increased in the H and M heifers, but the response was delayed in the L group heifers. When the leptin concentrations were aligned to the week of puberty, in all treatments plasma leptin concentrations increased with time but did not differ ($P>0.10$) among treatments for the 4 weeks before to 2 weeks after puberty. Plasma leptin 'pulse characteristics' are shown in Figure 6.6. Unlike the characteristic pulsatile nature, of LH secretion, the leptin 'pulses' were less sharply defined. Compared to M or L groups heifers, the H group heifers tended ($P<0.10$) to have greater pulse frequency at 8 mo, and larger pulse amplitude ($P<0.01$) at 10 mo of age.

There was a significant treatment x time interaction ($P < 0.05$) for plasma glucose and insulin concentrations (Figure 6.8 A, B) which were, in general, lower for the L compared to the H groups. Plasma NEFA concentrations exhibited considerable variability; were higher in the L relative to the H groups but were reduced with time and after changing to the phase-2 diet in all groups (Figure 6.8 C). There was a significant treatment x time interaction ($P < 0.05$) for plasma GH and IGF-1 concentrations (Figure 6.9 A, B). Plasma GH concentrations exhibited considerable variability but treatment differences were apparent by 11 weeks after the start of the study, with the concentrations being elevated ($P < 0.05$) in the L realtive to the H or M heifers. As for NEFA, GH concentrations were reduced in all groups after changing to P2 diet. Plasma IGF-1 concentrations were rapidly increased ($P < 0.01$) in the H and M heifers within 4 weeks compared to L heifers, and followed a similar pattern to leptin during the peripubertal period.

6.4. Discussion

Among the factors that determine the onset of puberty, nutrition plays a critical role (Sejrsen, 1994; Schillo et al., 1992). It is well recognized that puberty is delayed in undernourished heifers. In the present study we attempted to alter the timing of puberty by manipulating dietary energy and protein density and controlling feed allowance in Phase 1 of the experiment. Therefore, as expected, the average daily gain differed between treatments and this was reflected in differences in the onset of puberty. Slowly growing heifers attained puberty at an older age than rapidly growing heifers, with heifers on the P1M, P2L-12 and P1L diets being older at puberty by 1.5, 5 and 7 mo respectively compared to P1H heifers. The age at which P1H and P1M heifers attained puberty is in close agreement with other studies employing similar growth rates (Radcliff et al., 1997; Niezen et al., 1996; Adams et al., 1994). Despite these age differences, the body weight at puberty was similar among treatments ranging from 270 to 330 kg. This is consistent with the hypotheses that puberty occurs at a critical body weight (Kennedy and Mitra, 1963).

We are not aware of studies that evaluated body composition changes in Holstein heifers during the peripubertal period. To quantify changes in body reserves through pubertal transition we used the urea dilution technique for an indirect in vivo assessment of body composition in conjunction with BCS and ultrasound measurements of back-fat thickness. Estimates of body fat from urea space ranged from 2 to 12%, and estimates of body protein ranged from 18 to 20%, which are comparable to fat and protein estimates from other laboratories (Tomlinson et al., 1997; Steen et al., 1992). When compared at similar chronological ages, heifers on the P1L diet had lower percentages of body fat and protein relative to P1H or P1M heifers (Figures 6.2 and 6.3). When compared at a common physiological stage, which in this study was puberty, neither the percentages of body fat and protein nor back-fat thickness differed among treatments (Table 6.2). This would imply that heifers require a certain level of both body fat and protein reserves to attain puberty; however, our data should be confirmed utilizing larger numbers of animals and a direct proximate analysis of body composition at puberty in Holstein

heifers. However, at puberty, P1L and P2L-12 heifers appeared leaner with lower BCS values. The relative amounts and rates of fat deposition at various sites might have differed among treatments in our study. It is likely that P1L and P2L-12 heifers deposited more internal fat relative to subcutaneous fat. Such an effect may have been reflected in differences in BCS with the P1L and P2L-12 heifers appearing leaner and taller, but the technically more precise ultrasound measurements of back-fat thickness failed to detect significant differences between treatments. Further, of the variables considered, ADG, BW and body fat percent together explained 98 % of the variation in age at puberty.

Collectively, our data support the theory that in addition to body weight, adequate reserves of body fat and protein are essential for the attainment of puberty in dairy heifers. This is consistent with the 'critical body weight' (Kennedy and Mitra, 1963) and 'critical body fat' hypotheses (Frisch, 1980). Assuming that body composition at any fraction of mature weight is constant (Owens et al., 1995) and that puberty occurs at 55% of mature size in Holstein heifers (NRC, 2001), it could be argued that body composition at puberty should be relatively constant if mature weight is unaltered by dietary manipulation. However, other studies have shown that the body composition at puberty differed in beef heifers subjected to different nutritional regimens (Simpson et al., 1998; Hall et al., 1995; Yelich et al., 1995). In these studies, heifers were older (approximately 8 months of age) and heavier (about 220 kg BW) at the initiation of the experiment compared to our study. Heifers in our study might have been relatively immature physiologically at the initiation of the experiment, and also were subjected to more chronic dietary treatments compared to other reports. It is likely that the metabolic, endocrine and somatic adaptations to long-term treatments might differ from relatively short-term nutritional manipulations. In addition to the level and duration of nutritional treatments, breed differences might explain some of the discrepancies between studies. For example, in contrast to beef breeds, Holsteins were reported to have less body fat at similar empty body weight (NRC, 2000) and deposit more internal fat (Owens et al., 1995). It is also conceivable that the urea dilution technique that we used in this study may be less accurate in estimating body fat in Holstein breeds compared to beef breeds

which have greater adipose reserves. Regardless, both the urea space and back-fat values lend support to the hypothesis that a certain level of tissue reserves are essential for transition through puberty.

The hormonal and/or metabolic signals that gauge the level of body reserves and convey this information to the brain to trigger puberty in dairy cattle are not clearly understood. For a hormone/metabolite to be a pubertal signal, the plasma concentrations should remain unaltered irespective of nutritional conditions. In this study, independent of dietary manipulation, plasma leptin concentrations increased with the approaching puberty. Recent reports in beef heifers support our contention (Garcia et al., 2002, 2003). Thus, we provide here the first evidence that leptin fulfills such a role and could be an ideal candidate for initiating the onset of puberty in dairy heifers.

Concomitant with somatic adaptations, rapid maturation of the hypothalamic-pituitary-gonadal axis occurs at puberty. A cardinal sign of increased hypothalamic-pituitary activity is increased LH secretion as first ovulation approaches (Kinder et al., 1995; Evans et al., 1994; Day et al., 1987). Though the mean LH concentrations and pulse frequency did not differ among treatments at 8 mo of age, LH pulse amplitude was greater in P1H and P1M heifers compared to P1L heifers (Figure 6.6). Retrospective examination of ovarian maps and plasma progesterone concentrations revealed that P1H and P1M heifers were approximately 55 and 90 d before first ovulation when frequent blood sampling for LH was done at 8 mo of age. In beef heifers, the number of LH pulses was reported to be relatively constant until 50 d before puberty followed by a significant increase until first ovulation (Melvin et al., 1999; Evans et al., 1994; Day et al., 1987). This increase is coincident with a reduction in estradiol negative feedback on LH secretion (Melvin et al., 1999; Day et al., 1987). Therefore, it is possible that the strong inhibitory effect of estradiol on hypothalamic GnRH might override any potential influence of relatively mild dietary restriction on the hypothalamic pituitary axis until 50 d prepuberty. If true, this might explain our inability to detect treatment differences in LH pulse frequency at 8 mo of age. The tendency for increased pulse amplitude in P1H and P1M heifers at 8 mo relative to 10 mo of age concurs with the high amplitude pulses observed in beef heifers at 90 d prior to first ovulation (Evans et al., 1994). The marginal

numerical increase in mean LH concentrations at 8 mo, despite a significant increase in pulse amplitude, might have a causal effect in increasing the number of large follicles (> 10 mm) and the size of the dominant follicle in both P1H and P1M heifers compared to P1L heifers. In addition to LH, changes in metabolic signals such as insulin and IGF-1 (Armstrong et al., 2001; Ireland et al., 2000) might also have supported the increased number and size of large follicles. However, a confounding factor in comparing dietary effects at a similar chronological age is the influence of physiological maturity on follicular dynamics and LH profiles.

Prepubertal beef heifers fed energy dense diets were reported to have larger dominant follicles at a younger age but the size of the ovulatory follicle at puberty was similar to that of heifers fed low energy diets (Bergfeld et al., 1994). Similarly, in our study, the maximum size and growth rate of the non-ovulatory dominant follicle increased with age but did not differ between P1H and P1M heifers at puberty. This increase in both size and growth rate of the dominant follicle might be a consequence of strong support provided by the high frequency- low amplitude LH pulses, and is consistent with other reports (Melvin et al., 1999; Evans et al., 1994). However, the maximum size of the first-ovulatory follicle was larger in P1H compared to P1M heifers despite lack of significant differences in LH profiles between the treatments. This is suggestive of the influence of local ovarian effects of metabolic signals such as IGF-1, IGF-binding proteins or other factors (Armstrong et al., 2001; Bossis et al., 2000; Ireland et al., 2000).

We are not aware of studies that evaluated the effects of realimentation of feed-restricted heifers on peripubertal follicular dynamics. In this study first pubertal ovulation occurred in P2L-12 heifers approximately 47 ± 3 d after switching from the low diet in Phase 1 to the common Phase 2 diet. This is consistent with studies in anestrous beef heifers, wherein resumption of cyclicity occurred after 50 (Rhodes et al., 1995) to 80 days (Bossis et al., 2000) following realimentation. Compared to P1M heifers, in P2L-12 heifers the nonovulatory dominant follicle and the first and second ovulatory dominant follicles were larger, while the peak progesterone levels and CL growth were lower in the first cycle. It is possible that a reduction in negative feedback effect of progesterone on

the hypothalmic-pituitary axis could have resulted in increased LH secretion (Boland et al., 2001; Ireland et al., 2000), which in turn might have supported the increased size of the first ovulatory follicle. Alternatively, the progesterone concentrations found in the first cycle could be a bye-product of our twice weekly sampling frequency rather than an event of physiological significance. Apart from LH, increases in plasma IGF-1 during realimentation might also have contributed to the large size and rapid growth of the first and second ovulatory follicles in P2L-12 heifers.

In summary, we have described changes in body composition and ovarian follicular dynamics in dairy heifers during the pre and peripubertal period. Our data support the concept that puberty in dairy heifers occurs at a constant body composition and body weight independent of growth rate. Further, independent of dietary manipulation, plasma leptin concentrations increased with the approaching puberty. We contend that leptin could be the signal that relays information on body reserves to the brain to initiate puberty in dairy heifers. Puberty was delayed in heifers fed low, compared to either medium or high energy and protein-dense diets, or low group heifers realimented to a common postpubertal diet. This indicates that age at puberty is pliable and is determined to a large extent by the plane of nutrition. The early onset of puberty in high and medium diet group heifers was associated with the occurrence of high frequency low amplitude LH pulses. We have also demonstrated that dietary energy and protein density has a significant influence on peripubertal follicular dynamics in dairy heifers. At a similar chronological age, heifers fed nutrient dense diets had larger dominant follicles compared to low group heifers. However, realimentation of heifers fed diets low in energy and protein during the peripubertal period resulted in the first ovulation of a follicle which was larger than that of medium group heifers but similar in size to high group heifers. The relative consistency in body composition at puberty, as reported here, warrants further investigation into the hormonal and metabolic messengers that relay information on somatic reserves and activate the hypothalamic-pituitary-gonadal axis. The significant impact of nutrient density on follicular dynamics, as demonstrated in this study, underscores the need for further research on the influence of

different feeding regimens on ovarian function in dairy heifers during the pre- and peripubertal periods.

Table 6.1. Ingredient and chemical composition of diets.

Composition	Phase 1 diets			Phase 2 diet
	High (P1H)	Medium (P1M)	Low (P1L)	
Ingredient (% of DM[1])				
Barley silage	31.8	36.6	48.3	28.2
Alfalfa silage	-	-	-	28.2
Grass hay	-	-	-	14.1
Alfalfa hay	23.9	32.6	40.9	-
Rolled barley	19.8	17.2	10.6	29.4
Canola meal	7.8	6.4	-	-
Corn gluten meal	13.5	5.5	-	-
Tallow	2.9	1.5	-	-
Fortified mineral salt[2]	0.1	0.1	0.1	0.1
Vitamin ADE premix[3]	0.1	0.1	0.1	0.1
Chemical composition (Mean ± SEM)				
DM (%)	59.3 ± 1.0	57.4 ± 1.2	52.9 ± 1.4	52.9 ± 1.8
OM[4] (% of DM)	90.7 ± 0.2	90.8 ± 0.2	90.4 ± 0.3	90.1 ± 0.2
CP[5] (% of DM)	20.9 ± 0.7	18.1 ± 0.5	13.5 ± 0.2	13.9 ± 0.4
Crude fat (% of DM)	4.6 ± 0.2	3.4 ± 0.1	1.6 ± 0.1	2.1 ± 0.1
NDF[6] (% of DM)	44.1 ± 1.2	47.8 ± 1.3	54.3 ± 1.2	48.9 ± 1.0
ADF[7] (% of DM)	25.7 ± 0.9	28.3 ± 1.2	33.6 ± 1.1	29.0± 1.0
Lignin (% of DM)	6.2 ± 0.3	6.9 ± 0.4	8.5 ± 0.4	6.7 ± 0.7
Ash (% of DM)	9.3 ± 0.1	9.2 ± 0.1	9.6 ± 0.1	9.9 ± 0.1
ME[8] (Mcal/kg DM)	2.62	2.42	2.28	2.29
NE_g[9] (Mcal/kg DM)	0.97	0.93	0.85	0.86

[1]Dry matter

[2]Contained 37% Na, 58% Cl, 80 mg CO/kg, 400 mg Cu/kg, 200 mg I/kg, 6000 mg Mn/kg, 400 mg Se/kg, 800 mg Zn/kg; Champion Feed Services Ltd., Edmonton, AB, Canada.

[3]Contained 3,846 KIU Vitamin A/kg, 2,292 KIU Vitamin D/kg, and 6,539 IU Vitamin E/kg, Champion Feed Services Ltd., Edmonton, AB, Canada.

[4]Organic matter; [5]Crude protein; [6]Neutral detergent fiber; [7]Acid detergent fiber; [8]Metabolizable energy; [9]Net energy of gain.

Table 6.2. Effect of dietary energy and protein density on average daily gain, age, body weight, body condition score, back-fat thickness, empty body fat and protein percent, and growth measurements at puberty.

	P1H	P1M	P1L	P2L-12
Age (mo)	9.27 ± 0.49^{a}	10.8 ± 0.47^{b}	16.54 ± 0.75^{c}	14.13 ± 0.67^{d}
Average daily gain (kg/d)	1.04 ± 0.02^{a}	0.83 ± 0.02^{b}	0.52 ± 0.03^{c}	0.65 ± 0.03^{d}
Body weight (kg)	281.6 ± 12.1	282.4 ± 11.5	316.2 ± 18.2	309.2 ± 16.2
Body condition score	3.36 ± 0.08^{a}	3.20 ± 0.07^{ad}	2.56 ± 0.11^{b}	3.02 ± 0.10^{cd}
Back-fat thickness (mm)	3.36 ± 0.23	2.83 ± 0.22	2.55 ± 0.34	2.68 ± 0.31
Empty body fat (%)	7.41 ± 0.34	7.17 ± 0.34	6.97 ± 0.48	5.86 ± 0.59
Empty body protein (%)	19.25 ± 0.05	19.09 ± 0.05	19.15 ± 0.07	18.98 ± 0.08
Growth measurements (cm):				
Height at withers	109.6 ± 1.5^{a}	112.3 ± 1.4^{a}	119.2 ± 2.2^{b}	113.7 ± 2.2^{a}
Height at hip	113.9 ± 1.4^{a}	116.5 ± 1.3^{a}	121.7 ± 2.1^{b}	119.0 ± 2.1^{a}
Body length	92.3 ± 2.1	91.6 ± 2.1	97.7 ± 3.2	92.2 ± 3.2
Heart girth	150.7 ± 2.6^{a}	152.4 ± 2.5^{a}	162.2 ± 3.9^{b}	163.2 ± 3.9^{b}

LS Means ± SEM with different superscripts within a row differ ($P < 0.05$).

Table 6.3. Ovarian follicular characteristics in prepubertal heifers at 8 mo of age based on ultrasonographic ovarian scans over a 21 d period. Heifers were fed either high (P1H), medium (P1M), or low (P1L) energy and protein dense diets in Phase 1 of the study.

	P1H	P1M	P1L
Class 1 follicles (<5 mm)	21.4 ± 4.2	17.6 ± 3.4	17.7 ± 3.4
Class 2 follicles (6-10 mm)	3.3 ± 0.6	2.9 ± 0.5	4.4 ± 0.5
Class 3 follicles (>10 mm)	0.9 ± 0.1[a]	0.8 ± 0.1[a]	0.5 ± 0.1[b]
Nonovulatory dominant follicle:			
Lifespan (d)	17.0 ± 1.3	18.2 ± 1.1	16.5 ± 1.1
Maximum size (mm)	12.83 ± 0.62[a]	12.22 ± 0.27[a]	10.56 ± 0.51[b]
Growth rate (mm/d)	0.76 ± 0.18	0.72 ± 0.14	0.84 ± 0.14
Regression rate (mm/d)	0.82 ± 0.15	0.89 ± 0.13	0.79 ± 0.12
Subordinate follicle:			
Maximum size (mm)	8.25 ± 0.73	8.67 ± 0.60	8.00 ± 0.60
Growth rate (mm/d)	0.90 ± 0.26	0.46 ± 0.24	0.46 ± 0.22
Regression rate (mm/d)	0.57 ± 0.13	0.61 ± 0.11	0.38 ± 0.11

LS Means ± SEM with different superscripts within a row differ ($P < 0.05$).

Table 6.4. Ovarian follicular characteristics in heifers during the peripubertal period. Heifers were fed either high (P1H) or medium (P1M) energy and protein dense diets in Phase 1 of the study. Transrectal ultrasonography was performed for 50 d at 10 mo of age (P1H, P1M), and for 70 d at 12 mo after changing from low to a Phase 2 diet (P2L-12).

	P1H	P1M	P2L-12
Class 1 follicles (< 5 mm)	25.6 ± 4.3^{a}	22.3 ± 3.9^{a}	39.5 ± 4.3^{b}
Class 2 follicles (6 - 10 mm)	5.5 ± 0.8	4.2 ± 0.7	5.6 ± 0.8
Class 3 follicles (> 10 mm)	0.9 ± 0.1	0.9 ± 0.1	1.1 ± 0.1
Nonovulatory dominant follicle:			
Lifespan (d)	19.30 ± 1.12	19.89 ± 1.03	20.56 ± 1.12
Maximum size (mm)	13.30 ± 0.30^{ab}	13.09 ± 0.27^{a}	14.01 ± 0.30^{b}
Growth rate (mm/d)	0.93 ± 0.05^{ab}	0.77 ± 0.06^{a}	1.07 ± 0.06^{b}
Regression rate (mm/d)	0.90 ± 0.07	0.86 ± 0.06	0.84 ± 0.07
Subordinate follicle:			
Maximum size (mm)	8.40 ± 0.43	8.08 ± 0.39	8.43 ± 0.43
Growth rate (mm/d)	0.47 ± 0.09^{a}	0.66 ± 0.08^{a}	0.74 ± 0.09^{b}
Regression rate (mm/d)	0.61 ± 0.07	0.62 ± 0.06	0.63 ± 0.07
First ovulatory dominant follicle:			

Lifespan (d)	12.25 ± 1.02	9.80 ± 0.91	10.00 ± 0.91
Maximum size (mm)	14.10 ± 0.53 [a]	12.50 ± 0.53 [b]	14.70 ± 0.53 [a]
Growth rate (mm/d)	0.61 ± 0.14 [a]	0.91 ± 0.13 [ab]	1.27 ± 0.13 [b]
Second ovulatory dominant follicle:			
Lifespan (d)	10.50 ± 2.61	11.50 ± 2.61	9.60 ± 2.34
Maximum size (mm)	14.12 ± 1.06 [ab]	11.87 ± 1.06 [a]	14.90 ± 0.95 [b]
Growth rate (mm/d)	0.97 ± 0.16	0.99 ± 0.16	1.31 ± 0.15
Third ovulatory dominant follicle:			
Lifespan (d)	10.00 ± 2.83	14.00 ± 1.41	12.00 ± 2.83
Maximum size (mm)	15.00 ± 1.31	14.00 ± 1.31	12.87 ± 0.66
Growth rate (mm/d)	1.25 ± 0.10	0.83 ± 0.10	0.82 ± 0.05

LS Means ± SEM with different superscripts within a row differ ($P < 0.05$).

Table 6.5. Characteristics of corpus luteum (CL) and plasma progesterone concentrations in the first and second ovulatory cycle of heifers fed either high (P1H), or medium (P1M) energy- and protein dense diets in Phase 1. Ovaries were scanned ultrasonically for 50 d starting at 10 mo of age in P1H and P1M heifers, and for 70 d starting at 12 mo of age in P2L-12 heifers after changing from a low to a Phase 2 diet.

	P1H	P1M	P2L-12
First ovulatory cycle:			
CL characteristics			
Lifespan (d)	8.67 ± 2.63	10.00 ± 2.28	10.00 ± 2.04
Maximum size (mm)	14.88 ± 2.51	15.92 ± 2.17	12.30 ± 1.95
Growth rate (mm/d)	0.92 ± 0.13[a]	1.17 ± 0.10[a]	0.34 ± 0.10[b]
Regression rate (mm/d)	0.41 ± 0.25	0.61 ± 0.22	0.82 ± 0.31
Plasma progesterone (ng/mL)			
Mean concentration	1.59 ± 0.59	2.09 ± 0.46	1.36 ± 0.51
Peak concentration	4.10 ± 0.35[a]	3.75 ± 0.38[a]	1.90 ± 0.66[b]
Interovulatory interval (d)	11.33 ± 4.17	11.20 ± 3.23	11.60 ± 3.23
Second ovulatory cycle:			
CL characteristics			

Lifespan (d)	14.67 ± 2.89	16.67 ± 2.89	17.00 ± 2.24
Maximum size (mm)	21.07 ± 2.76	17.23 ± 2.76	20.21± 2.14
Growth rate (mm/d)	0.90 ± 0.63	0.80 ± 0.63	0.81 ± 0.49
Regression rate (mm/d)	0.61 ± 0.32	0.68 ± 0.32	1.12 ± 0.28
Plasma progesterone (ng/mL)			
Mean concentration	3.10 ± 0.51	2.24 ± 0.46	2.41 ± 0.59
Peak concentration	4.99 ± 0.34	5.24 ± 0.36	4.09 ± 0.48
Interovulatory interval (d)	24.00 ± 2.52	23.00 ± 2.52	20.50 ± 1.26

LS Means ± SEM with different superscripts within a row differ ($P < 0.05$).

Figure 6.1. Schematic overview of experimental design. Solid lines represent Phase 1, and broken lines represent Phase 2 of the study. Frequent blood sampling for determining LH characteristics was done at 8 and 10 mo of age from a subset (n = 6) of P1H, P1M, and P1L heifers. Ovarian ultrasonography (hatched boxes) was performed at 8 mo, 10 mo (P1H, P1M, and P1L), and at 12 mo (P2L-12) of age.

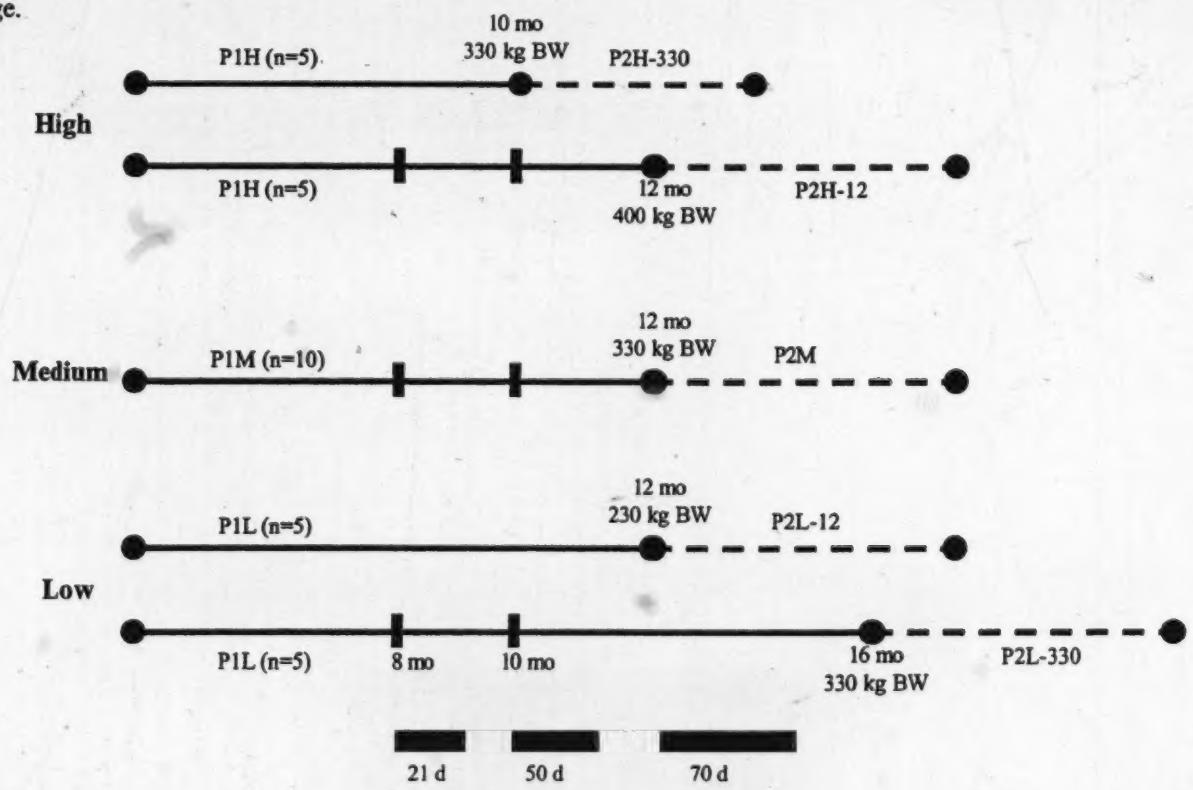


Figure 6.2. Changes in estimated body fat (panel A) and body protein (panel B) percent from 8 to 12 mo in heifers fed high (P1H -■-), medium (P1M -▲-) or low (P1L -●-) energy and protein dense diets. No differences (P > 0.10) were detectable between P1H and P1M treatments. Differences within a month between P1L and P1H or P1M are indicated by asterisk (s) (*P < 0.05; **P < 0.01). Arrows indicate the approximate age at puberty in High and Medium group heifers.

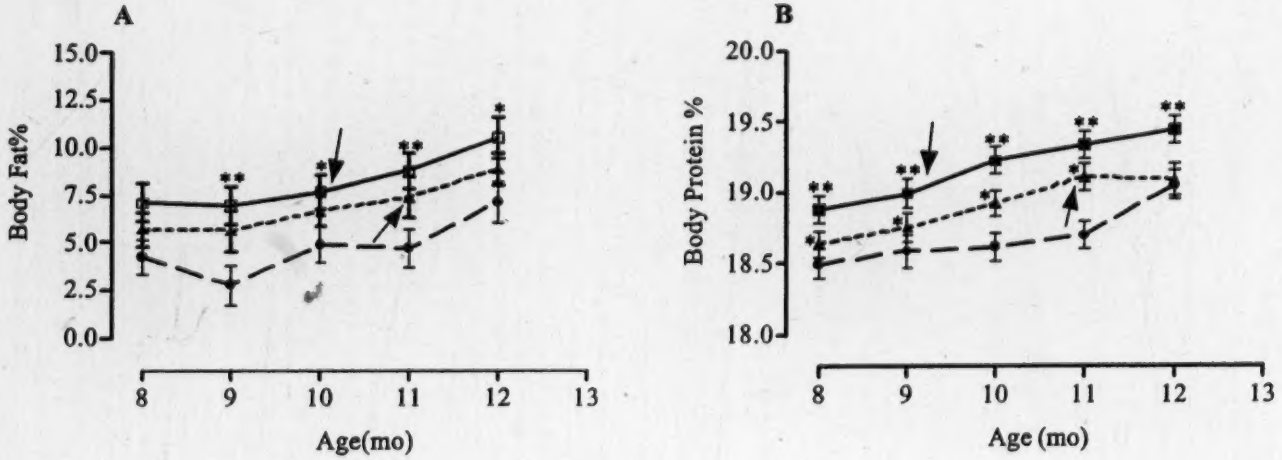


Figure 6.3. Panel A: Diameter profile of nonovulatory dominant follicle at 8 mo of age in heifers fed high (P1H -■-), medium (P1M -▲-) or low (P1L -●-) energy and protein dense diets. A quadratic regression equation ($P < 0.0001$) was used to estimate diameter of the nonovulatory dominant follicle. Panel B: Diameter profiles of the nonovulatory dominant follicle prior to first ovulation, and the first ovulatory follicle in heifers fed diets varying in energy and protein density. A quadratic regression equation ($P < 0.0001$) was used to estimate the diameter of the nonovulatory dominant follicle, and a linear regression equation ($P < 0.0001$) was used to estimate the diameter of the first ovulatory follicle in high (-■-), medium (-▲-) and P2L-12 heifers (-◆-).

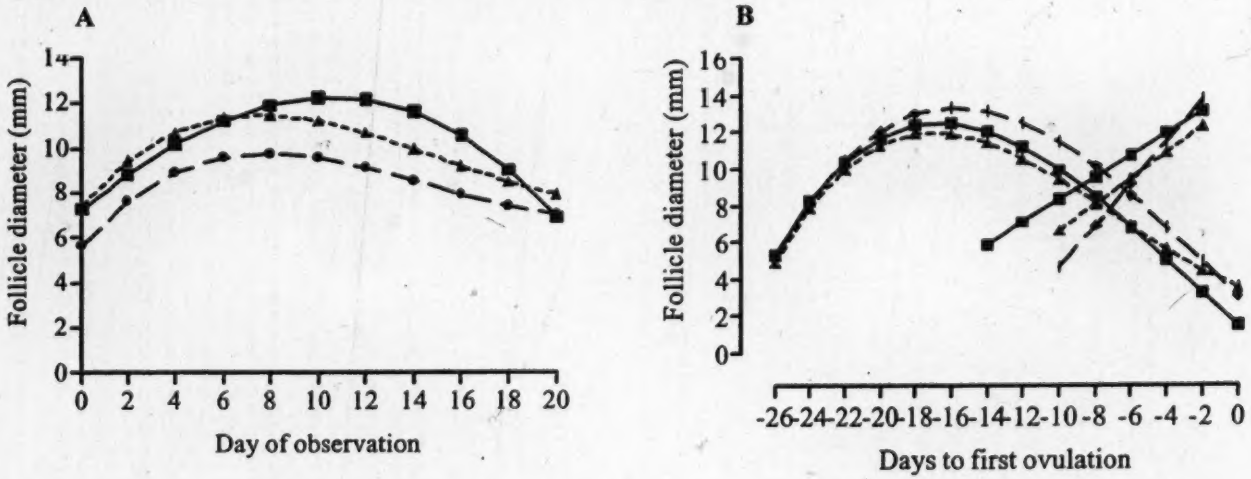


Figure 6.4. Effect of feeding high (P1H ■), medium (P1M □) or low (P1L ▨) energy and protein dense diets on plasma LH characteristics in dairy heifers. Blood was sampled at 15 min frequency for 8 h at 8 and 10 mo of age in a subset of heifers (n = 6/treatment). Data presented at 10 mo represents 3 P1H, 5 P1M, and 6 P1L prepubertal heifers. Mean (A) and basal (B) concentrations of LH, LH pulse frequency (C) and amplitude (D) are shown (LS Means ± SEM). For a given age, means with different superscripts are different ($P < 0.05$).

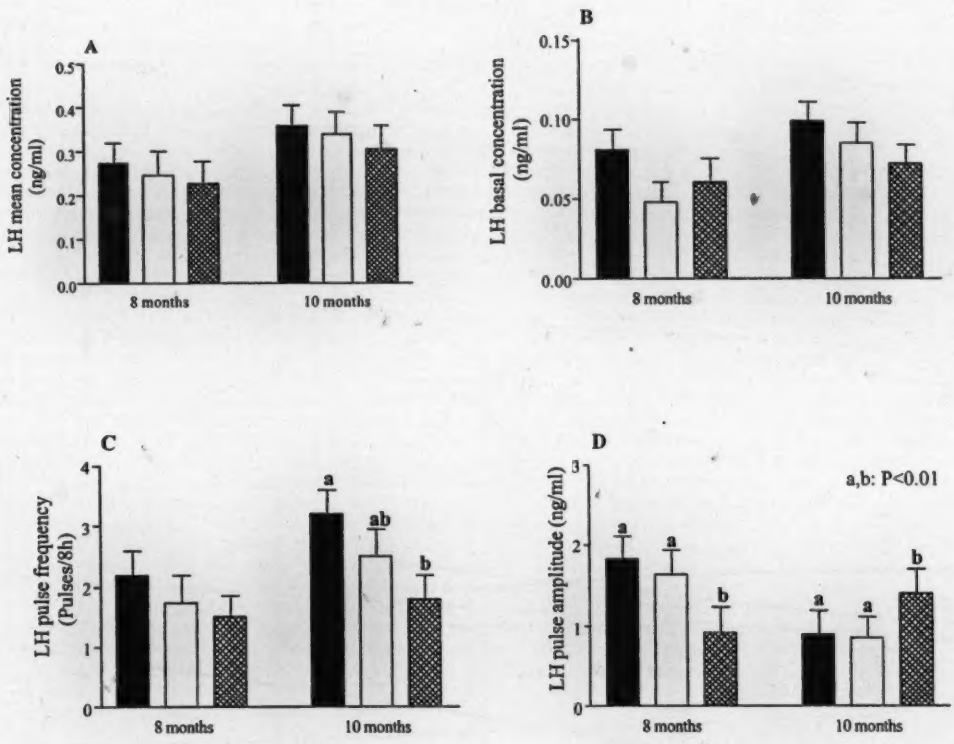
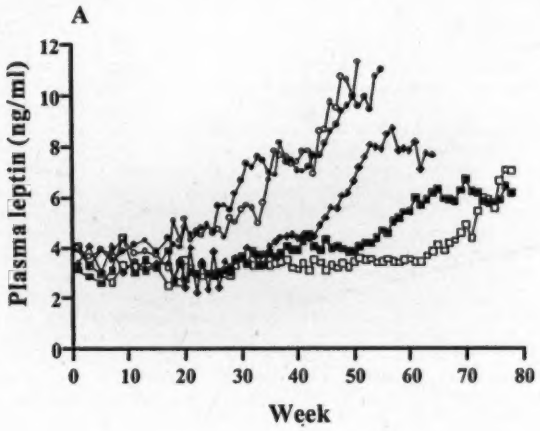


Figure 6.5. Plasma leptin concentrations (ng/ml) in heifers fed diets varying in energy and protein density. Panel A: Heifers (n=10/treatment) were fed diets of either high (P1H), medium (M1H; ◆) or low (L1H) energy and protein density during the pre and peripubertal period of phase 1. Half the heifers within the high and low groups entered phase-2 (P2) at either 12 months of age (P2H-12 ●; P2L-12 ■) or at 330 kg body weight (P2H-330 ○; P2L-330 ❑). Panel B: Plasma leptin profiles relative to the week of puberty (week 0). There were no differences ($P > 0.10$) between subgroups of heifers on the high diet, hence the data were combined and represented as a single group (●).



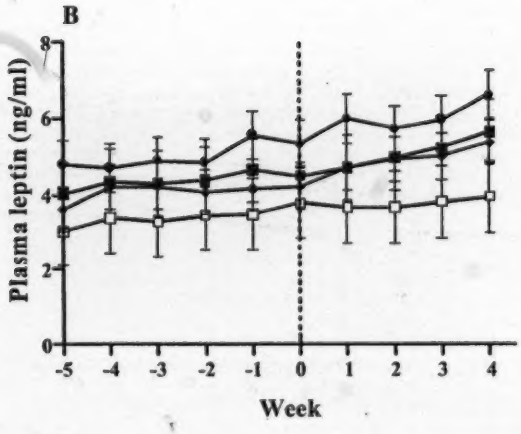


Figure 6.6. Effect of feeding high (P1H ■), medium (P1M □) or low (P1L ⊠) energy and protein dense diets on plasma leptin characteristics in dairy heifers. Blood was sampled at 15 min frequency for 8 h at 8 and 10 mo of age in a subset of heifers (n = 6/treatment). Data presented at 10 mo represents 3 P1H, 5 P1M, and 6 P1L prepubertal heifers. Mean (A) and basal (B) concentrations of leptin, leptin pulse frequency (C) and amplitude (D) are shown (LS Means ± SEM). For a given age, means with different superscripts are different ($P < 0.05$).

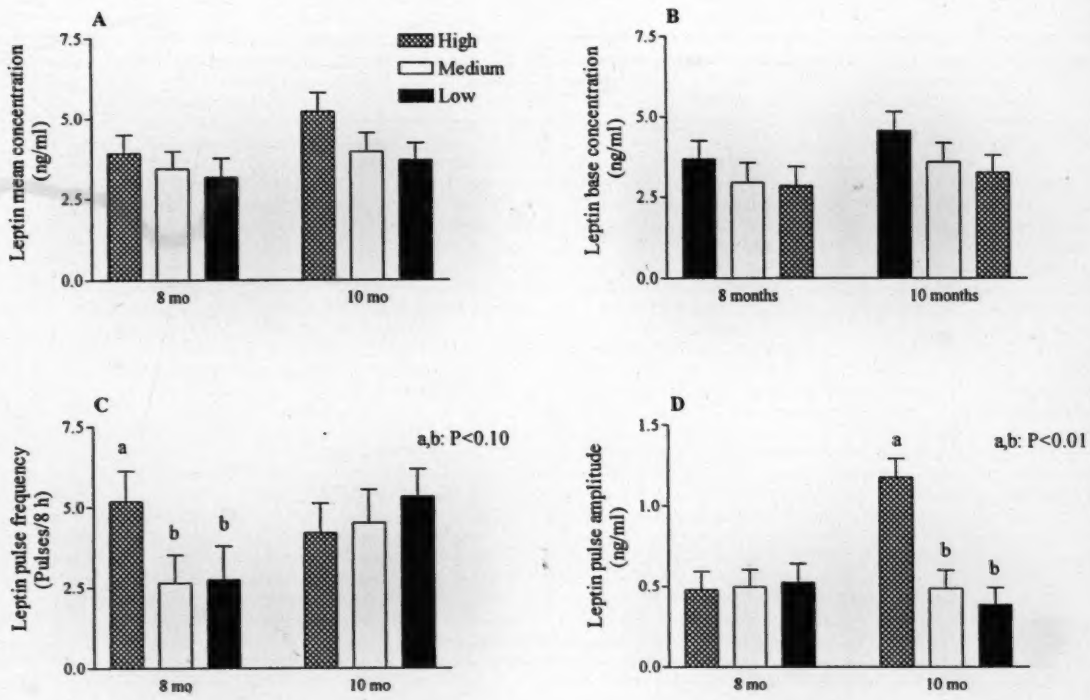


Figure 6.7. Changes in body weight (panel A) and back-fat thickness (panel B) in heifers fed diets varying in energy and protein density. Heifers (n=10/treatment) were fed diets of either high (P1H), medium (M1H; ◆) or low (L1H) energy and protein density during the pre and peripubertal period of phase 1. Half the heifers within the high and low groups entered phase-2 (P2) at either 12 months of age (P2H-12 ●; P2L-12 ■) or at 330 kg body weight (P2H-330 ○; P2L-330 ❑).

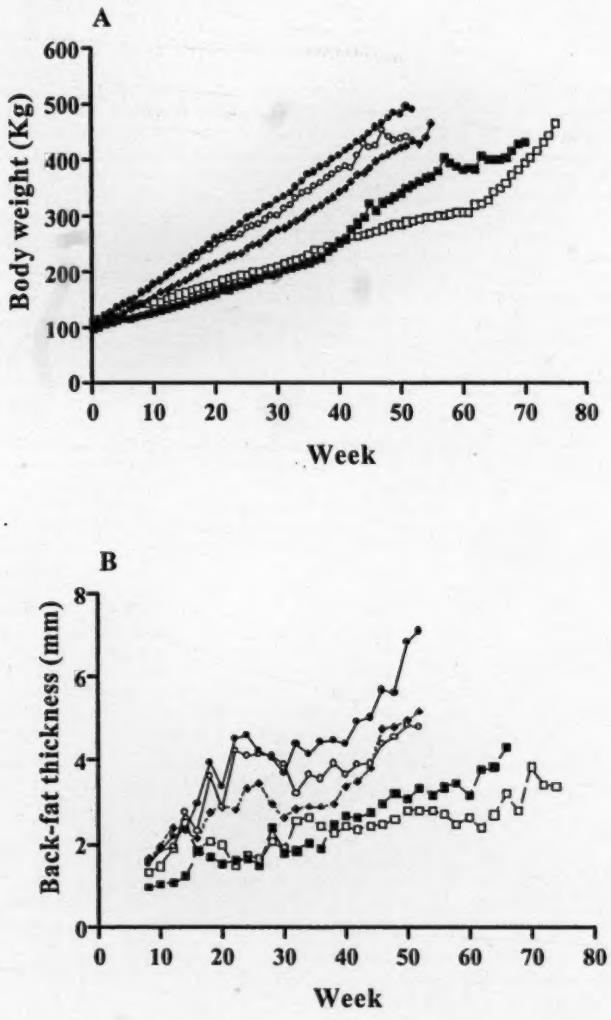


Figure 6.8. Changes in plasma concentrations of glucose (A), insulin (B), nonestreified fatty acids (NEFA, C) in heifers fed diets varying in energy and protein density. Heifers (n=10/treatment) were fed diets of either high (P1H), medium (M1H; ◆) or low (L1H) energy and protein density during the pre and peripubertal period of phase 1. Half the heifers within the high and low groups entered phase-2 (P2) at either 12 months of age (P2H-12 ●; P2L-12 ■) or at 330 kg body weight (P2H-330 ○; P2L-330 ❑).

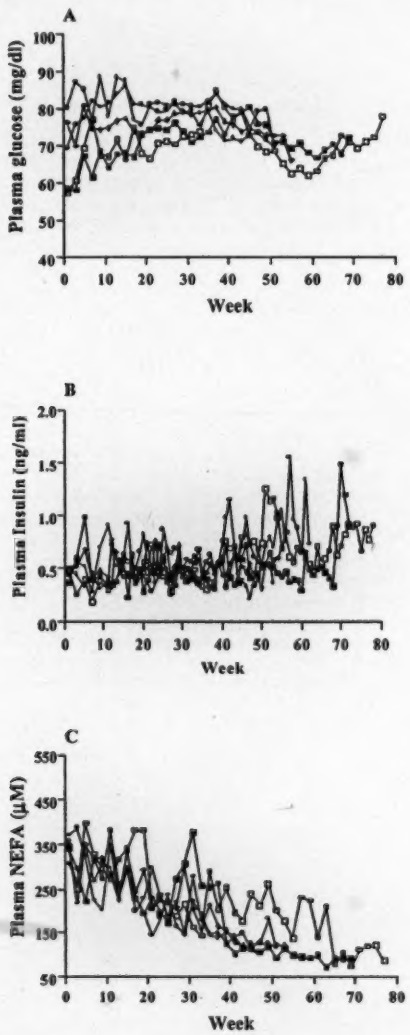
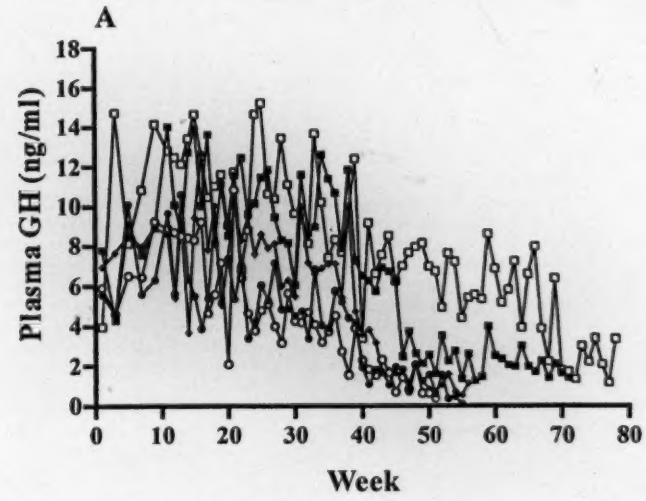
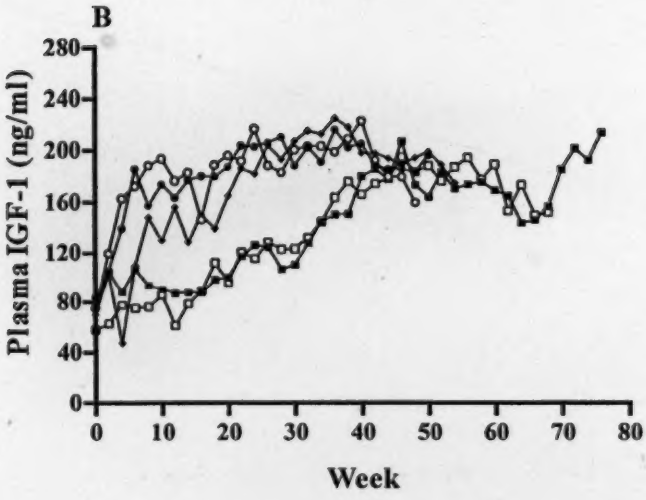


Figure 6.9. Changes in plasma concentrations of growth hormone (GH; A) and insulin-like growth factor-1; B) in heifers fed diets varying in energy and protein density. Heifers (n=10/treatment) were fed diets of either high (P1H), medium (M1H; ◆) or low (L1H) energy and protein density during the pre and peripubertal period of phase 1. Half the heifers within the high and low groups entered phase-2 (P2) at either 12 months of age (P2H-12 ●; P2L-12 ■) or at 330 kg body weight (P2H-330 ○; P2L-330 ❑).





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CHAPTER 7
General discussion

7.1. Introduction

Leptin, a hormone secreted primarily from adipose tissue, has proved to be the messenger that relays information on the status of body fat reserves to the brain to regulate food intake and puberty in humans and rodents. Although, it is well known that body fat reserves play a crucial role in regulating feed intake and reproduction in dairy cattle, the putative signal (s) that acts as a connecting link has remained elusive to date. Thus, the discovery of leptin has opened up an exciting area in dairy cattle research. In order to understand the physiology of leptin in cattle, it is necessary to gain information on the response of leptin to dietary manipulation. Nutritional regulation of leptin expression and release is known largely from studies in humans and rodents. To date, very few studies have evaluated the effects of nutrition on leptin in ruminants. The overall objectives of this work were to identify the tissues expressing leptin and its receptors; determine the effects of short-term feed deprivation on plasma concentrations of leptin; determine the role of macronutrients in the short-term regulation of leptin secretion; and determine the effects of long-term dietary manipulation on circulating leptin concentrations during the peripubertal period in dairy cattle.

7.2. Detection of mRNA for leptin and leptin receptors in tissues of cattle

As a first step towards understanding the physiological significance of a hormone, it is necessary to identify the tissues where the hormone and its cognate receptors are expressed. Using an RT-PCR based approach, the expression of mRNA for bovine leptin, the long- (Ob-Rb) and short- (Ob-Ra) forms of the bovine leptin receptor was determined in central and peripheral tissues of Holstein cattle, and presented in Chapter 2. Leptin mRNA was detectable in mammary parenchyma and in adipose tissue with similar transcript abundance among the subcutaneous, pericardial, perirenal, and mesenteric adipose depots. The Ob-Rb mRNA was detectable in all four adipose depots,

mammary parenchyma, semitendanosus muscle, liver, adrenal cortex, spleen, kidney, testis, mesenteric lymph node, lung, aorta, abomasum, duodenum, jejunum, ileum, hypothalamus, pituitary, brain stem, cerebral cortex, cerebellar cortex, pons, and pineal gland. The Ob-Ra mRNA was detectable in the liver, adrenal cortex, spleen, pituitary, and brain-stem, but not in the other tissues surveyed.

Unlike rodents and humans, the almost ubiquitous distribution of the long-form of the leptin receptor in the calf tissues implies that leptin may have multiple physiological functions in the bovine. However, an inherent limitation of our RT-PCR based method is that it does not provide information on the type of cells expressing the receptor. *In situ* hybridization and immunocytochemistry would be advantageous in identifying the cellular localization of leptin receptor mRNA and protein, respectively. Nevertheless, the wide spectrum of tissues expressing the leptin and leptin receptor mRNA should provide a foundation for future studies on quantifying the transcript abundance and understanding downstream signalling events of the leptin receptors in response to nutritional and or/hormonal manipulation.

7.3. The response of plasma leptin concentrations to short-term feed deprivation is dependent on the physiological state and body condition of dairy cattle

As described in detail in Chapter 1, a major function of leptin appears to be to act as a signal of adaptation to fasting. In the second set of studies (Chapter 3), designed to identify the putative changes in leptin with feed deprivation, it was demonstrated that plasma leptin concentrations were acutely responsive to fasting and refeeding in dairy cattle. The response, however, differed among cattle at various physiological stages with the fasting-induced reduction in plasma leptin being blunted in non-lactating pregnant cows and heifers compared to early lactation cows. It is unlikely that leptin is involved in mediating postfast satiety in cattle. On the contrary, the fasting-induced acute hypoleptinemia in early lactating cows could be an adaptive response to increase feed intake during the refeeding period. The positive correlation of leptin with changes in plasma glucose, insulin, and IGF-1 suggest that these signals might mediate the effects of fasting on leptin production in cattle.

The changes in leptin with short-term feed deprivation indicate that the hormone is responsive to short-term nutritional manipulation. In the next two studies, the effects of specific macronutrients on regulation of leptin production was determined.

7.4. The mRNA abundance and plasma leptin concentrations are unaffected whereas both transcript abundance and plasma cholecystokinin concentrations are increased by canola oil supplementation to dairy cattle

Dietary fat supplementation often produces hypophagic effects depending on the amount of fat fed. The aim of the third study was to determine whether dietary fat supplementation affects leptin expression and release, and to determine the relative importance of leptin and cholecystokinin in medaiting the hypophagic effects of fat supplementation. In this study we compared the effects of feeding versus abomasal infusion of canola oil on leptin and cholecystokinin (CCK) gene expession and release. Given the significant impact of unsaturated fat on nutrient digestibility and milk composition, we also determined the nutrient digestibility and milk composition of cows receiving relatively large amounts of canola oil.

Feeding oil did not exert any adverse effects on nutrient digestibility but resulted in increased intestinal flows of biohydrogenation intermediates such as *trans*-11 18 : 1 and *cis*-9 *trans*-11 18 : 2 (CLA) and their subsequent transfer to milk. In contrast to the effects of dietary canola oil, abomasal infusion of oil resulted in reductions in feed intake and nutrient digestion. Both feeding and infusion treatments stimulated CCK gene expression in the duodenum and elevated plasma CCK concentrations. However, the data do not support a role for CCK in mediating satiety through an endocrine mechanism of action. It is speculated that CCK might be acting either through paracrine and/or neurocrine routes to influence feed intake in cattle. Both feeding and infusion treatments had no effect on the mRNA abundance of leptin, lipoprotein lipase, or acetyl-coA carboxylase in adipose tissue. Plasma concentrations of leptin, insulin and IGF-I were also not altered, indicating that these signals may not be involved in mediating the short-term hypophagic effects of dietary fat. Plasma concentrations of oleic acid and linoleic acid were significantly greater with infusion compared to feeding oil or the control diet to

cows. Thus, the hypophagic effects of supplemental fat in cattle is dependent on the amount of unsaturated fatty acids reaching the intestine and this satiety effect may be mediated through CCK, oleic acid and (or) linoleic acid, but leptin is not involved.

In this study the animals were relatively thin-conditioned, in late lactation, and the body weight and body condition score were not affected by treatments. If the leptin response to fat supplementation is dependent on body fat reserves it may be reasoned that cows in good body condition will exhibit a robust response compared to thin conditioned animals. The next study tested this hypothesis.

7.5. Response of plasma leptin concentrations to jugular infusion of glucose or lipid is dependent on the stage of lactation of Holstein cows

As part of the homeostatic adaptations to lactation, dairy cows mobilize large reserves of body fat to support milk production in early lactation and gain body fat reserves concomitant with declining milk production in late lactation. We utilised this lactation-dependent differences in body fat reserves, and the associated hormonal and metabolic changes, to determine whether the response of circulating leptin to macronutrient supply is dependent on body condition and stage of lactation. Therefore, in this study (Chapter 5), we investigated the hormonal and metabolite responses to isoenergetic jugular infusions of glucose or lipid in early and late-lactation Holstein cows.

With the exception of a hypophagic effect of lipid infusion in late lactation, feed intake was unaffected by other treatments. Glucose infusion resulted in hyperglycemia and hyperinsulinemia which were greater in late compared to early lactation cows. Plasma leptin and IGF-1 concentrations were unaffected by glucose treatment in early lactation but were increased in late-lactation cows. In early lactation cows, plasma leptin, insulin and IGF-1 were unresponsive to lipid treatment, despite a marked increase in plasma NEFA, BHBA and a reduction in GH concentrations. In comparison to the delayed leptin response to glucose, the stimulatory effect of lipid infusion on leptin secretion in late-lactation cows was relatively rapid and occurred in the absence of any significant changes in plasma insulin, IGF-1 or GH. It is proposed that insulin-mediated

glucose metabolism maybe involved in the stimulatory effects of glucose on leptin secretion in late lactation cows but that the stimulatory effects of lipid are direct and independent of insulin or IGF-1. In early lactation animals a strong inhibitory effect of GH on leptin expression and release, in addition to low adipose reserves, might override any short-term stimulatory effect of glucose or lipid on leptin secretion.

Clearly both glucose and lipid have significant impact on leptin secretion in dairy cows in late-lactation. Future studies on the response of leptin to the degree of unsaturation and chain length of fatty acids and/or the amount of starch supplied to the intestine, and the intracellular events involved in these responses, would be valuable in understanding the the role of macronutrients on leptin secretion. Further, studies using hyperinsulinemic or hyperglycemic clamps should also prove useful in delineating the role of glucose and insulin on leptin production in dairy cattle.

7.6. Dietary energy and protein density affects plasma leptin concentrations in dairy heifers during the peripubertal period

All the above studies evaluated the short-term changes in leptin production. The purpose of this study was to evaluate the leptin response to long-term nutritional manipulation using a heifer model, and describe the changes in body composition, plasma leptin, and ovarian follicular dynamics in dairy heifers during the pre and peripubertal period (Chapter 6). The data support the concept that puberty in dairy heifers occurs at a constant body composition and body weight independent of growth rate. Further, independent of dietary manipulation, plasma leptin concentrations increased with approaching puberty. It is contended that leptin could be the signal which relays information on body reserves to the brain to initiate puberty in dairy heifers. The early onset of puberty in heifers fed high or medium energy- and protein- dense diets was associated with the occurrence of high frequency low amplitude LH pulses. It was also demonstrated that dietary energy and protein density has a significant influence on peripubertal follicular dynamics in dairy heifers. At a similar chronological age, heifers fed nutrient-dense diets had larger dominant follicles compared to undernourished heifers. The significant impact of nutrient density on follicular dynamics, and the

interesting changes in plasma leptin concentrations during the peripubertal period, as demonstrated in this study, underscores the need for further research on the influence of different feeding regimens on ovarian function and leptin concentrations in dairy heifers during the pre- and peripubertal periods. Studies on the direct effects of leptin administration are also needed to determine whether leptin can advance the onset of puberty in dairy heifers.

7.7. Future research

As mentioned earlier, leptin receptors are expressed in a wide array of central and peripheral tissues in cattle. The obvious question that arises is whether these receptors have a functional role. As a first step towards delineating the functional significance of these receptors, it is necessary to identify the cell types that express the receptors. For example, as mentioned in Chapters 1 and 2, leptin is believed to play a role in lipid handling in the gut of rodents and humans. To test this in ruminants, a preliminary study, maybe conducted using tissue samples collected from various regions of the gastrointestinal tract. Rather than collecting such samples at the abattoir, ideally the adult animals must be euthanized at the research center for harvesting tissues. Each sample can then be partitioned and processed for both immunohistochemistry as well as snap-frozen in liquid nitrogen for later quantification of leptin receptor expression along the gut by real-time RT-PCR. Once the techniques have been validated with this preliminary study, the next step could be to determine the functional significance of the leptin receptors. To address this objective an *in vitro* model using cultures of different regions of the gut may be appropriate. Under such controlled *in vitro* culture conditions, the effects of fatty acids varying in chain length and unsaturation on the abundance of leptin receptors by real-time RT-PCR, as well as changes in DNA binding activity of STAT's using electrophoretic mobility shift assays, could be determined. However, it must be realized that a potential limitation with such an approach is that an alteration in STAT-induced DNA binding could be mediated by other hormones as well. Nonetheless, such a model should prove to be useful in understanding whether leptin is involved in lipid absorption by the gut.

In the study described in Chapter 2, prepubertal male Holstein calves were utilized to study leptin and leptin receptor expression. Some of the questions that need to be addressed are: Is the expression in female calves similar to male calves, or is there a sexual dimorphism?, and Is there a developmental change in leptin and leptin receptor expression?. To address these questions, a developmental study could be conducted utilizing female Holstein calves. The animals would be euthanized at strategic points in time for harvesting tissues. A plausible design might involve sacrificing the calves (n=4/time point) at birth, and at 3, 6, 12, 18, and 24 months of age. These time-points correspond approximately to the various stages of mammary growth and reproductive tract development, assuming an averge daily gain of 0.8 kg/d. The tissues should be processed for both gene expression and immunocytochemistry, and thus could be valuable for analysis of not only leptin but for a host of other developmental signals. Blood samples would also be collected simultaneously for hormone and metabolite assays.

As described in Chapters 3 and 5, clearly body condition is an important determinant of leptin response to nutritional stimuli. The signals responsible for the dramatic fall in leptin with fasting in early lactation cows and the delayed response in late-lactation cows require further investigation. In this context, an ideal model for determining the role of glucose and insulin might involve the use of a hyperglycemic and/or hyperinsulinemic clamp techniques to prevent the fasting-induced reduction in circulating leptin concentrations. Given the potential for carry-over effects with fasting experiments, a completely randomized design with a 2 x 2 factorial arrangement of treatments (Control: Fed; Control + Hyperglycemic clamp; Fasting; Fasting + Hyperglycemic clamp) may be appropriate. Following a 3-week rest period, the experiment could be repeated with the use of a hyperinsulinemic clamp. A similar approach could also be utilized to determine whether infusion of volatile fatty acids could prevent the reduction in leptin with fasting. To determine whether the differential leptin response to fasting in early and late-lactation animals is due to differences in sympathetic nervous system activity, administration of β_2 /β_3 antagonists to prevent the fall in leptin, using a similar design as described above, might be useful.

In Chapter 5, the importance of body condition in modulating the leptin response to fat supplementation has been described. The direct stimulatory effects of lipid on leptin production warrants further research into understanding the molecular events involved. A role for the hexosamine pathway in mediating the effects of fat on leptin secretion was proposed and needs to be verified. A latin-square design similar to the one described in Chapter 5 could be adopted with adipose and muscle tissue biopsies at the end of each period. In addition to quantifying the abundance of transcripts for leptin and leptin receptor in these tissues, the expression of transcriptional regulators of leptin expression such as peroxisome proliferator activated receptors and adipocyte determination and differentiation-dependent factor-1, and the transcript abundance and activity of glutamine:fructose-6-phosphate aminotransferase (the rate-limiting enzyme of hexosamine biosynthetic pathway) need to be determined. As an alternative to the above mentioned *in vivo* model, the effects of specific fatty acids on leptin expression and release may also be studied using explant cultures of adipocytes from early and late-lactation cows.

A direct role for leptin in the regulation of feed intake, reproduction, and lipid metabolism in cattle would invariably require direct administration of the hormone. As described in Chapter 6, leptin appears to play a role in initiating the onset of puberty in dairy heifers. To confirm this hypothesis, studies are needed to determine whether leptin can advance the onset of puberty. The ability of exogenous leptin to advance puberty in undernourished heifers would provide a strong support to our hypothesis that leptin initiates the onset of puberty in dairy heifers. However, a major limitation for the conduct of such studies is the requirement of relatively large amounts of bovine leptin.

The field of leptin physiology in cattle is still in its infancy. Therefore, some of the above mentioned research ideas are by no means exhaustive and should be considered only as starting points for formulating and testing valid hypotheses.

7.8. Conclusions

In conclusion, leptin expression is restricted to adipose tissue whereas leptin receptors are expressed in a range of central and peripheral tissues in cattle. Plasma

concentrations of leptin are acutely responsive to short-term feed deprivation and the response is particularly rapid in early-lactation animals. In the short-term, fat supplementation does not influence plasma leptin concentrations in thin condition animals, whereas cows in good body condition respond to fat as well glucose supplementation. In the long-term, dietary energy and protein density has a significant impact on plasma leptin concentrations and leptin may act to initiate the onset of puberty in dairy heifers.

Appendix-1: Isolation of RNA from adipose tissue biopsies using TRIzol reagent

(from Beswick NS, Kennelly JJ. J. Anim Sci. 2000; 78: 2412-2419)

1. Thaw TRIzol to room temperature; place mortar & pestle at -80^0C to cool.
2. Record weight of 14 ml labelled Falcon® (Beckton & Dickinson) tubes (3 tubes /per sample).
3. Grind tissue using the pre-chilled mortar & pestle on a bed of dry ice. Add liquid N_2 to the sample to keep cold and facilitate easy grinding.
4. Weigh ~ 300-500 mg of ground tissue into each of the preweighed 14 ml tubes.
5. Immediately add 5ml of TRIzol to each tube – vortex to suspend the sample. The time from weighing to addition of Trizol should be very short.
6. Homogenization: Rinse the smaller homogenizer probe with sterile d.d. H_2O and soak the probe in 0.1 N NaOH, again rinse with sterile d.d. H_2O. Homogenize at maximum speed for < 45 sec. Rinse the probe between samples with sterile d.d. H_2O. Vortex the homogenized samples briefly.
7. Allow samples to stand at room temperature for ~30 min.
8. Centrifuge at 12,000 x g (9500 rpm- Beckman JA 20.1 rotor) for 10 min at 4^0C.
9. Label a new set of tubes and add 1 ml of $CHCL_3$ to each tube (i.e. @ 0.2 ml/ml TRIzol).
10. Carefully pierce the fat layer with a sterilized pasteur pippette and transfer the supernatant from the spun tubes into the tubes containing $CHCL_3$. Vortex briefly for 5 sec.
11. Let stand at room temperature for 5 - 10 min.
12. Centrifuge at 12,000 x g (9500 rpm- Beckman JA 20.1 rotor) for 15 min at 4^0C.
13. Label a new set of tubes and add 2.5 ml of Isopropyl alcohol to each tube (i.e. @ 0.5 ml/ml TRIzol).
14. Transfer the clear supernatant from the spun tubes into the tubes containing Isopropyl alcohol.
15. Incubate: either at room temperature for 1 – 2 h (or) overnight at -20^0C.

16. Centrifugation: Add 1ml of the solution repeatedly into 1.5ml eppendorf tubes and spin at 12,000 x g for 10 min at 4^0C. Discard supernatant and repeat addition to collect the RNA pellet at the bottom of the eppendorf tubes.
17. Wash pellet 2 times with 1ml of 75% Ethanol by centrifuging. Let pellet air-dry for < 5 min.
18. Resuspend the pellet in 50 µl of DEPC® treated H_2O. Determine RNA concentration & store at -80^0C.

Appendix-2: Leptin RIA Procedure

1.1 Buffer- PABET = (PROTEIN ASSAY BUFFER w/ EDTA w/ Tween-20)

0.15 M NaCl (8.8 g/L)

0.01 M PO_4 (1.2 g/L) = use only sodium phosphate monobasic, anhydrous)

0.01% sodium azide (0.1 g/L)

0.1% Knox unflavored gelatin (1 g/L)

0.01 M EDTA (tetrasodium salt; 3.2 g/L)

0.05% Tween 20 (500 µl/L)

Adjust pH to 7.1 to 7.2 with 10 NaOH.

1.2 Preparation of polypropylene Iodogen tubes

1) Mix Iodogen as follows: 10 mg iodogen dissolved in 2 ml Chloroform

2) Add 100 µg iodogen/polypropylene "bullet" tube (i.e. 20 µl/tube)

3) Air dry

4) Store capped in freezer in the dark.

1.3 Leptin Standard

1) 20 ng/ml in PABET

2) Preparation of leptin standard: – Take 148ug of the original ovine leptin received from Gertler and solubilized it in 740 µl H_2O, then take 40 µg (200 µl) and dilute this in 1000 ml of PABET.

1.4 Preparation of ^{125}I – Leptin

1) Prepare 30 µg Leptin in 50 µl H_2O

2) Need 1 reaction vial prepared with 100 µg iodogen

3) Add 30 µg of oLeptin to the iodogen vial

4) Add ~500 to 750 µCi Na ^{125}I (5 to 7.5 µl) and react for 5 minutes

5) Count reaction vial (with the ^{125}I) to determine transfer (a crude count is ok via a survey meter)

6) Transfer contents of the reaction vial to a 10 cm AcA54 column (Sigma cat # U8628) and collect 0.5 ml fractions (the setting on the fraction collector with a 18 gauge needle is 35 drops = 0.5ml) and fractions 15 to 18 are the good fractions (see below). This column type is critical.

7) Count reaction vial after transfer to determine transfer percent

8) Count 10 µl aliquots of elution fractions to determine elution profile

9) Dilute the desired fraction in PABET to achieve ~25,000 cpm/100 µl

1.5 Primary antibody (1st Antibody)

Antibody #7137 and dilute it to achieve a final dilution of 1:30,000. To make 250 ml add 83.25 µl of 1ost Ab 7137 + 247.5 ml PABET

1.6 Secondary antibody (2nd Antibody)

Sheep anti-rabbit that has been pre-precipitated

1.7 Assay Procedure

1st Incubation – Add standards or sample (200 µl) and buffer and 1st antibody and incubate at 4°C for 24 hours.

2nd Incubation – add 100 µl ^{125}I – Leptin and incubate at 4°C for 20 hours

3rd Incubation – add 100 µl 2nd Antibody and incubate at room temperature for 15 minutes then add PBS and spin at 3500 rpm for 30 min. Discard supernate and count pellet.

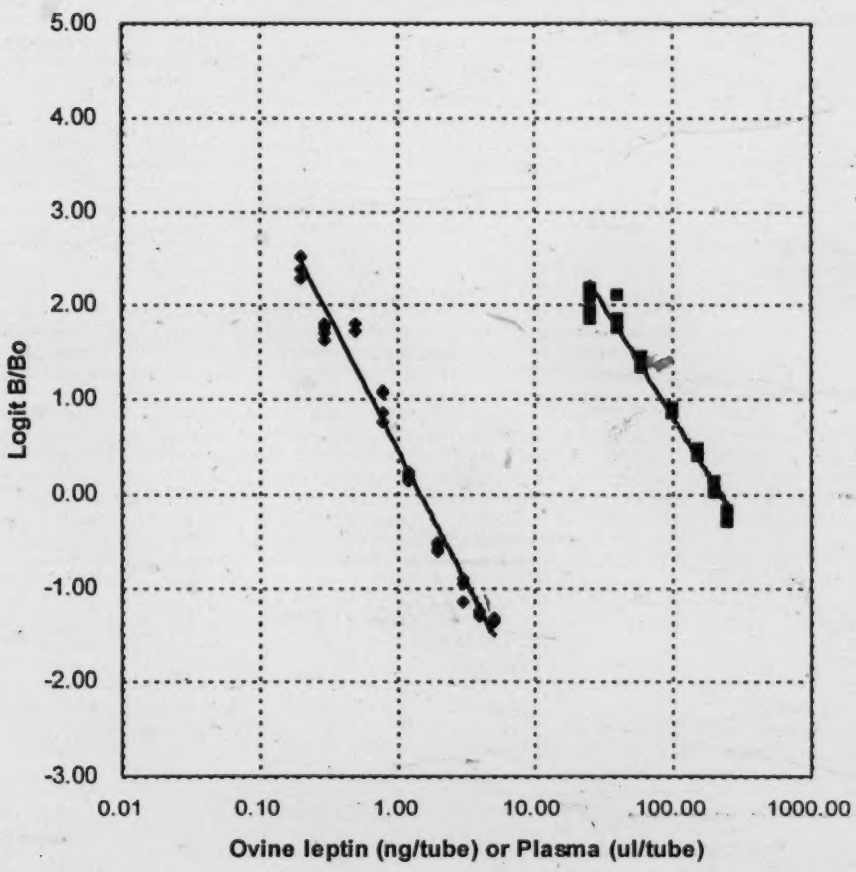
1.8 Reference:

Delavaud C, Bocquier F, Chilliard Y, Keisler DH, Gertler A, Kann G. Plasma leptin determination in ruminants: effect of nutritional status and body fatness on plasma

leptin concentration assessed by a specific RIA in sheep. J Endocrinol 2000; 165: 519-26

1.9 Leptin parallelism

Linear log-logit representation of ovine leptin standard curve (◆). Displacement of ^{125}I-labelled ovine leptin with 30 to 250 µl of bovine plasma (■) resulted in curves that were parallel to the ovine leptin standard curve.



Appendix-3: Bovine Insulin RIA Procedure

2.1 Reagents and Products:

1) Bovine Insulin (Sigma Cat# I-5500; Lot # 128F-023)
2) 1st Ab: Guinea pig Anti-Bovine Insulin Serum (Cat#: 4010-01; Cad $223/Vial/1000 tubes; Cedarlane Laboratories Ltd, Ontario Canada): Reconstitute with 1 ml double-distilled H_2O, aliquot into 100 µl volumes and freeze at -20^0C.
3) 2nd Ab: Goat Anti-guinea pig IgG ((GAGPG): Cat#: 2020; Lot# GP2023 (2P); Cedarlane Laboratories Ltd, Ontario Canada). Reconstitute with 100ml EDTA buffer and freeze at -20^0C in 10 ml volumes.
4) Normal Guinea Pig Serum ((NGPS): Cat# 566400; Lot# B41158; Calbiochem): Reconstitute with 5 ml double-distilled H_2O, aliquot into 400 µl volumes and freeze at -20^0C.
5) 0.01 M PBS Buffer: pH = 7.5

d.d.H_2O make-up volume	0.5 L	1L	2L	4L
Monobasic PO_4 ($NaH_2PO_4.H_2O$): (g) (0.74 mmoles/L)	0.051	0.1015	0.203	0.406
Dibasic PO_4 (Na_2HPO_4): (g) (9.26 mmoles/L)	0.658	1.3153	2.631	5.261
Sod. Azide (NaN_3): (g) (3.08 mmoles/L)	0.100	0.200	0.400	0.800
Nacl (g) (140 mmoles/L)	4.091	8.182	16.364	32.728

6) 0.05 M EDTA Buffer: Heat stir to dissolve; adjust pH to 7.5 after cooling to room temperature; store @ 4^0C:

Above PBS +EDTA (g):	9.306	18.612	37.224	74.448

7) 1% BSA/PBS: Made fresh weekly by spraying 1 g BSA/100ml PBS buffer and let standing for ~15-30 min.

2.2 Summary of Protocol:

Day	Item	Volume	Bench dilution	Buffer
1	Sample +Buffer	(100+400µl)=500µl	Ab1=1:1600	BSA
	Ab1 in NGPS	100µl	NGPS = 1:200	EDTA
2	Label	100µl	10,000 cpm/100µl	BSA
3	GAGPG	1 ml	1:10	EDTA
	Total Volume =	1.7 ml		

2.3 Assay Protocol

Day 1:

1. Preparation of standard curve:

a) Weigh 10 mg of insulin powder and dissolve in 50 ml of 0.012 N Hcl (Concentration =10 mg/50 ml = 0.2 mg/1ml = 20 µg/100µl = 1 µg/5 µl)

b) Making 20, 000 ng/ml stock: Dilute the stock (1 µg/5 µl = 200,000 ng/ml) 10x to get 20, 000 ng/ml [1:10; 0.1:1]. Measure 1ml BSA buffer, remove 0.1 ml, add 0.1ml of 1 µg/5 µl stock.. Vortex.

c) Making 1,000 ng/ml stock: Dilute the above 20, 000 ng/ml stock 20x to get 1,000 ng/ml stock. Will need ~ 6 ml of this for all assays. [1:20; x:6; x=0.3 ml]. Measure 6 ml of BSA buffer, remove and add 0.3 ml of the 20, 000 ng/ml stock. Vortex. Aliquot 1 ml into eppendorf tubes, label with reagent name (Binsulin), date, concentration (1000 ng/ml), volume (1 ml) and freeze at -20^0C. ***Preparation of stock solutions upto this step can be done well in advance of the actual assay.***

d) To prepare Stds' for 20 curves to be used in 2 weeks:

Total number of Stds = 4reps per std * 20 Stds =80

Volume of Std = 0.1 ml per tube * 80 Stds = 8ml * 2 for serial dilution = 16 ml or ~20 ml of highest Std.

To make highest std of 4.8 ng/0.1 ml, actual amount of Std needed is 960 ng [4.8 ng:0.1ml; x: 20ml; x =960 ng].

e) Making 100 ng/ml intermediate stock: Dilute 1000ng/ml stock 10x [1:10; x:10; x=1 ml]. Measure 10 ml BSA buffer, remove and add 1 ml of 1000 ng/ml stock. Vortex.

f) Making highest curve standard (i.e. 4.8 ng/0.1 ml): Need 960 ng in 2 ml BSA buffer. [100ng : 1ml; 960 ng:x; x =9.6 ml]. Measure 20 ml BSA buffer, remove and add 9.6 ml of 100 ng/ml intermediate stock to get the highest curve Std of 4.8 ng/ml. Therefore, now have 20 ml of 4.8 ng/0.1ml Std.

g) Preparing the standard curve by serial dilution: Label 9 tubes and add 10 ml of BSA buffer to all tubes. Serially dilute by taking 10 ml of the 4.8 ng/0.1ml Std and transferring sequentially. The range of the standard curve is: 4.8, 2.4, 1.2, 0.6, 0.3, 0.15, 0.075, 0.0375, 0.01875, and 0.009375 ng/ml. If running 3 mini-curves/assay, then the amount of Std needed = 0.1 * 4 reps * 3 = 1.2 ml/assay. Therefore, label 10 eppendorf tubes with the Std concentrations, aliquot ~1.4 ml/vial, and store at -20^0C.

2. Dilution of 1st Ab in NGPS:

a) Volume of NGPS needed = 0.1ml * 2500 tubes = 250 ml. NGPS final dilution =1:200 [1:200; x : 250; x =1.25 ml). Measure 250ml, remove and add 1.25 ml of stock NGPS. Add 0.1 ml to NSB tubes.

b) Volume of NGPS solution remaining = 250–(0.1*15)=240-1.5=248.5 ml.

c) Ab1 bench dilution: [1:1600; x:248.5; x = 0.155ml = 155 µl]. Remove and add 150 µl of stock Ab1 to the NGPS solution.

d) Working * assay tube =final Ab1 dilution (after label addition)
1/1600 * 0.1/0.7 = 1/11200

3. Add 100 µl of plasma sample in duplicate into 12 x 75 mm borosilicate glass tubes (Fisher Scientific). If handling large number of samples- keep the tubes in cold room (4^0C) after adding to ~ 500 tubes.

4. Parallelism : Need ~2.5 ml plasma for parallelism, recovery, and controls. Label 6 sets of tubes in duplicate as CP200, CP100, CP50, CP25, CP12.5, and CP 6.125. Add 200µl of plasma + 300µl of BSA buffer to CP200 tubes, and 100µl plasma + 400µl BSA buffer to CP 100 tubes. Add 1ml of BSA buffer to 4 tubes labelled as P50, P25, P12.5, and P6.125. Do 3 serial dilutions by adding 1ml plasma to P50 –mixing and transfering 1ml to P25, and so on. Transfer 100 µl from P50, P25, P12.5, and P6.125 tubes to corresponding CP50, CP25, CP12.5, and CP 6.125 tubes; and add 400 µl of BSA buffer to these tubes.
5. Recovery: Label 3 sets of tubes in duplicate as Blank, Spike-1, and Spike-2. Add 100 µl from the CP25 tubes to blank and spike tubes. To Spike-1 add 100 of µl Std 3 (0.0375 ng/ml) +300µl of BSA buffer; to Spike- 2 add 100µl of Std 5 (0.150 ng/ml) + 300µl of BSA buffer to give 500µl total volume.
6. Add 400 µl of BSA buffer to all sample and NSB tubes, except Totals.
7. Add 100 µl of Ab1 in NGPS to Std's, BM's, and sample tubes- EXCEPT NSB tubes. Cover with foil, vortex, and incubate overnight at 4^0C.

Day 2:

Dilute label in BSA buffer to give ~12000 cpm/100 µl. Cover the tubes with foil, vortex, and incubate for 20-24 h at 4^0C. Place some black racks and d.d H_2O in cold room (4^0C).

Day 3:

1. Dilution of 2^{nd} Ab (GAGPG) in EDTA buffer:

a) Reconstitution: Reconstitute 1 vial of freeze dried material with 100 ml EDTA buffer (1 vial/1000 tubes).

b) 3.34 gm% PEG: Weigh 3.34 gm of PEG 8000 (OMNIPUR, EMSCIENCE (Merck), Gibbstown, NJ, Cat# 6510). Make-up volume to 100 ml with EDTA buffer.

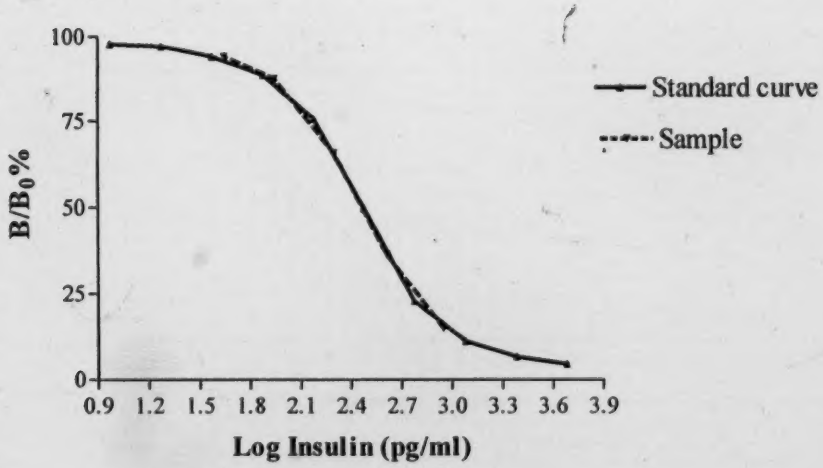
2. Add 1ml of 2^{nd} Ab to all except Total tubes in cold room (4^0C) with constant stirring. Takes ~45 min/2000 tubes. Vortex.
3. Incubate for 1-2 h at 4^0C. Transfer into black racks.

4. Centrifuge for ~45 min (or until aspiration) at 3000 rpm and 4^0C.
5. Aspirate and count for 2 min on the gamma counter.

2.4 Calculations:

1. Assay sensitivity = [(Mean B_{max} (cpm)) – (2*SD of B_{max} (cpm))/Mean B_{max} (cpm)] x 100; where B_{max} was the average of the zero binding standard tubes for that assay.
2. % Recovery = [(Concentration of spiked sample)/(ng added + Concentration of blank)] x 100
   where concentration is in ng/ml.
3. Intrassay CV% = Mean of assay error (or) Mean of %CV of controls within an assay.
4. Interassay CV% = % CV of controls among assays.

2.5 Parallelism:



Appendix-4: Isolation of RNA from intestinal biopsies using TRIzol reagent

(modified from Suominen et al., J Anim Sci 1998; 76: 2678-2686)

1. Composite 3-4 biopsy samples for each time-point, on dry ice, and record sample weight.
2. Add 200ul of TRIzol to the samples in Eppendorf microfuge tubes.
3. Homogenize samples (Caframo, Canlab, Ontario) for 45 sec at high speed (1200 rpm) using plastic pestles. To avoid cross contamination of samples, rinse the pestles with water followed by TRIzol. Place the samples on ice until all samples are homogenized.
4. Centrifuge for 30 sec at 5000g to precipitate all solids.
5. Add 200ul of TRIzol, homogenize and centrifuge as described in (3) & (4) above.
6. Add 400ul of TRIzol and homogenize as in (3) above.
7. Incubate all samples at room temperature for 15 min.
8. Add 160ul of Chloroform and vortex/shake vigorously for 15 sec.
9. Incubate at room temperature for 5 min.
10. Centrifuge at 12,000g for 15 min at ~4^{0}C.
11. Carefully transfer the upper aqueous phase into a new tube, without disturbing the interphase.
12. Add 400ul of isopropyl alcohol, vortex and precipitate overnight at -20^{0}C.
13. Centrifuge at 12,000g for 15 min at ~4^{0}C. Discard the supernatant.
14. Wash the pellet with 800ul of 75% ethanol by gentle vortexing.
15. Centrifuge at 7,500g for 5 min at ~4^{0}C.
16. Carefully remove the ethanol without disturbing the pellet.
17. Allow the pellet to air-dry for 5-10min, taking care not to completely dry the pellet.
18. Dissolve pellet in 50ul of DEPC water.
19. Determine the RNA concentration on a GeneQuant spectrophotometer (Pharmacia) at 260nm.
20. Store the RNA at -80^{0}C until further use.

Appendix -5: Rapid method for determination of total fatty acid content and composition of feedstuffs, digesta, feces, and plasma.

(modified from Sukhija PS, Palmquist DL. J.Agric. Food Chem. 1988; 36: 1202-1206)

5.1 Apparatus:

1. 150 x 25mm or 120 x 15mm Teflon-lined screw cap culture tubes for the one-step methylation procedure (A firm seal of cap and tube is essential for success).
2. 100 x 10mm screw-cap culture tubes for storing methyl esters in organic phase; 100 x 210mm screw cap culture tubes.
3. Water bath and vortexer.
4. Hewlett-Packard 5890 gas-liquid chromatograph fitted with automatic sampler 7673A, integrator 3392A, FID detector, and SP-2340 fused silica capillary column (0.32x3mm) (Supelco, Inc., Bellefonte, PA)

5.2 Reagents:

1. Benzene
2. Internal standard: Nonadecanoic acid (19:0)- 4mg/ ml in benzene, chloroform, or petroleum ether.
3. 5% Methanolic HCL: Freshly prepared by slowly adding adding 10 ml of acetyl chloride to 100 ml of anhydrous methanol.
4. 6% $K_2 CO_3$
5. Anhydrous sodium sulphate and Activated charcoal

5.3 Procedure

1. Accurately weigh 50-500 mg of dried feces, forage, or grain; or 100 mg of oilseed; or 100 µl plasma into preweighed culture tubes. (The amount of sample is selected to contain 10-50 mg fatty acids). Use 150 x 25mm culture tubes for feces and forages; for other samples 120 x 15mm tubes are more convenient.

2. To each tube add: 1 ml of benzene containing internal standard + 1mlof benzene +3ml of fresh 5% methanolic HCL slowly so that the solvents fall over the material without touching the side walls of the tube.
3. Tightly cap the tubes and vortex for 1min with a slow speed so that the material remains 2-3 cm from the bottom. Though vortexing is essential, it should be undertaken with caution because tranesterification may remain incomplete due to incomplete extraction if the sample spreads over the walls.
4. Heat the tubes for 2 h in water bath at 70^0 C. If solvent escaped, add 2ml of benzene after cooling and return the tube to the water bath to ensure complete methylation.
5. After the contents are cooled to room temperature, add 5 ml of 6% $K_2 CO_3$ and 2 ml of benzene. Acetyl chloride in the reaction mixture reduces the pH to 1-2; increasing the pH to neutrality is necessary to prevent degradation of the thin coating of the polyester stationary phase of GLC capillary columns.
6. Vortex the tubes at medium speed for ½ min, and centrifuge at 1500 rpm for 5 min.
7. Transfer the upper organic phase (benzene) with a Pasteur pipet to a 100 x 210 mm screw cap culture tube.
8. To the benzene extract add 1 g of anhydrous sodium sulphate and 1 g of activated charcoal (in case of presence of pigments).
9. Vortex the tubes for ½ min and allow to stand for 1h. Centrifuge the tubes for 5 min at 1500 rpm. Transfer the clear upper benzene layer containing methyl esters to a 100 x 10 mm culture tubes until analysis by GLC.
10. Gas chromatography conditions:

    Temperature: 160 to 180^0 C at 3^0 C/min for feed fats, and from 90 to 180^0 C for milk fat.

    Gas Flows: Carrier nitrogen 1ml/min; hydrogen 30 ml/min; air 400 ml/min.
11. Quantification of fatty acids:

    Total fatty acids (mg/gm of dry sample)

$$= \frac{\text{(total area under peaks)} - \text{(area under int. std)}}{\text{area of int std}} \times \frac{4\text{mg}}{\text{dry weight of sample (g)}}$$